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Genotoxicity and subchronic toxicity evaluation of Active Hexose Correlated Compound (AHCC)

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ABSTRACT

Active Hexose Correlated Compound (AHCC), a mushroom extract rich in α-1,4 linked glucans, is associated with immunostimulatory effects. AHCC is used in Japan as a dietary supplement to boost immune function and it also is purported to improve the symptoms of cancer and liver disease patients. A series of toxicological studies were conducted on a freeze dried preparation of AHCC (AHCC-FD) to further develop the body of evidence supporting the safety of this ingredient. AHCC-FD was not mutagenic to *Salmonella typhimurium* and did not exhibit clastogenicity in a mouse micronucleus assay. In a 90-day study, Sprague–Dawley rats were administered 1000, 3000, or 6000 mg/kg body weight/day by gavage. No changes attributable to AHCC-FD treatment were observed in overall condition, body weight, food consumption, ophthalmology findings, hematology and clinical chemistry parameters, and absolute and relative organ weights. Changes in urinary pH values observed in high-dose animals and mid-dose females were considered physiological rather than adverse effects given the acidic nature of AHCC-FD. Urinary protein also was increased in the same dose groups. As this finding was associated with decreased urinary pH and no evidence of kidney dysfunction was observed, it was considered of no toxicological significance. Histopathological changes related to AHCC-FD administration were observed in the limiting ridge of the stomach and in the liver of the high-dose group. The NOAEL was considered to be 3000 mg/kg body weight/day.

1. Introduction

Active Hexose Correlated Compound (AHCC) is a cultured mycelium extract obtained from *Lentinus edodes* of the *Basidiomycetes* family of fungi. The material is largely composed of various small molecular weight oligosaccharides (~5000 MW), of which 20–30% are α-1,4 hexose linked, and the sugar moiety of these linkages is partially acetylated. The product also contains other polysaccharides including β-1,3 glucans, and small amounts of protein, amino acids, lipids, and minerals. AHCC is available as a freeze-dried powder referred to as AHCC-FD (or AHCC powder) and as a fine granular product referred to as AHCC-Fine Granular (AHCC-FG; 60% AHCC-FD). AHCC-FG is essentially AHCC-FD powder that is reformulated with candelilla wax and microcrystalline cellulose additives.

Polysaccharides are ubiquitous among fungi from yeast to mushroom, and these compounds impart structural properties to the organisms. Although the polymeric compositions of various fungal polysaccharides are unique and specific to each organism, the structural configurations are highly conserved (Matsui et al., 2007). Due to the ubiquitous presence of fungus in the environment and diet, mammalian immune systems have developed innate pattern recognition systems for fungal polysaccharides. The interaction between fungal derived glucans and various cells of the immune system results in immunostimulatory effects, which in-turn prime the immune system for defense against potential invading microorganisms. The unique capacity of fungal derived glucans to act as biological response modifiers of the immune system has stimulated wide-spread research into their uses as functional foods.

The immunological effects of AHCC have been investigated in numerous publications, and the product has been utilized as an immunostimulatory food for over 15 years in Japan. AHCC has been reported to improve the prognosis of patients with postoperative hepatocellular carcinoma (Matsui et al., 2002), and improvements in the quality of life of patients with advanced liver cancer also have been reported (Cowawintaweewat et al., 2006). In studies conducted in rodents, AHCC has been shown to reduce metastasis of rat mammary adenocarcinoma (Matsushita et al., 1998), and to

Abbreviations: 2-AA, 2-aminoanthracene; 9-AA, 9-aminoacridine hydrochloride hydrate; AHCC, Active Hexose Correlated Compound; AHCC-FD, freeze-dried powder referred to as AHCC-FD; AHCC-FG, AHCC-Fine Granular; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, one-way analysis of variance; AST, aminotransferase; LOEL, lowest-observed-effect level; MMC, mitomycin C; NaN_3, sodium azide; NOAEL, no-observed-adverse-effect level; SD, Sprague–Dawley; WBC, white blood cell count.

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ameliorate the side-effects evoked by cisplatin chemotherapy in tumor-bearing mice (Hirose et al., 2007). AHCC also may find utility against viral and microbial infections, and has been reported to increase survival in rodents following various viral and bacterial challenges (Aviles et al., 2008; Nogusa et al., 2009; Ritz, 2008; Ritz et al., 2006; Wang et al., 2009).

The mechanism(s), and specific receptor mediated interaction(s) by which AHCC affects the immune system are not completely understood; however, studies in healthy subjects administered AHCC daily for a period of 4 weeks indicate that AHCC can modulate dendritic cell number and activity (Terakawa et al., 2008). Thus, the utility of AHCC in cancer subjects and in various infectious models in rodents may be mediated through its ability to stimulate dendritic cells, which are potent antigen presenting cells that are able to prime T-cells. Finally, recent evidence also has suggested that the functional properties of AHCC may be multi-factorial; in addition to direct modulation of the immune system, AHCC also has been observed to attenuate inflammation in rats with hapten-induced colitis *via* prebiotic effects on the colonic microflora (Daddaoua et al., 2007).

The safety of AHCC for use as a dietary supplement is partly supported by extensive anecdotal evidence obtained over 15 years of use as a supplement product in the Japanese marketplace, without reports of adverse effects. Recent human safety studies have been conducted in healthy subjects receiving daily quantities equivalent to 3 or 9 g of AHCC-FG over periods of four weeks to 14 days, respectively, with no evidence of toxicity, serious side-effects, or adverse changes in clinical chemistry or hematology endpoints (Spierings et al., 2007; Terakawa et al., 2008). Since the long-term safety of AHCC supplementation is limited to anecdotal evidence obtained from consumption of the product (3–6 g/day as AHCC-FG, equivalent to 1.8–3.6 g AHCC-FD) in Japan among unhealthy subjects, a subchronic toxicity study of AHCC-FD was conducted to determine the safety of AHCC for potential use as a functional food ingredient.

This report details studies evaluating the genotoxicity and oral toxicity of AHCC-FD. The genotoxicity of AHCC-FD was assessed using the bacterial reverse mutation assay and an *in vivo* mouse micronucleus test. A single dose study and a 90-day toxicity study were conducted in male and female Sprague–Dawley rats administered AHCC-FD *via* gavage.

2. Materials and methods[1,2]

2.1. Materials

Two formulations of AHCC are produced by Amino Up Chemical Co., Ltd. (Japan). The first is a highly water soluble hygroscopic deliquescent brown powder referred to as AHCC-FD. The second product is referred to as AHCC-FG, which is AHCC-FD powder that is coated with candelilla wax and microcrystalline cellulose additives. The use of candelilla wax renders the material insoluble in water and is intended to reduce digestion of the material during gastrointestinal transit. Product specifications for these products are presented in Table 1. In clinical practice, AHCC-FG is used with a typical daily recommended dose of 3 g (equivalent to 1.8 g AHCC-FD powder), although some physicians recommended up to 6 g (Amino Up Chemical Co., Ltd., personal communication). All clinical studies conducted to date on AHCC have utilized the AHCC-FG form of the product, with the exception of the study conducted by Spierings et al. (2007) wherein the liquid form of AHCC was studied (Amino Up Chemical Co., Ltd., personal communication).

All studies in this report were conducted using AHCC-FD. Two lots of AHCC-FD (supplied by the Amino Up Chemical Co., Ltd.) meeting product specifications (lots 43–0105 and 54–0618) were used for the Ames test. The mouse micronucleus assay, and single dose and repeat dose toxicity studies were conducted with the respective lots of 64–0606-1, N9603-1, and 83-0922-2. AHCC-FD is stable for up to two years when stored at room temperature in a well-closed container to avoid moisture.

2.2. Genotoxicity studies[3]

2.2.1. Ames assay

Two sets of experiments were conducted using the pre-incubation method. The first mutagenicity experiment utilized *Salmonella typhimurium* strains TA102, TA1535, and TA1537. Tester strain TA102 was obtained from Japan Industrial Safety and Health Association, Japan Bioassay Research Center. TA1535 and TA1537 were obtained from National Institute of Hygienic Science (current National Institute of Health Sciences). The S9 mixture was prepared from liver homogenates from Slc:SD male rats to which phenobarbital and 5,6-benzoflavone had been intraperitoneally administered for enzyme induction.

Based on the results of a dose-finding test, the highest concentration of the test substance in the main test was set at 5000 μg/plate, and a total of six concentrations (5000, 2500, 1250, 625, 313, and 156 μg/plate) were used in both the assays (*i.e.*, with and without metabolic activation). Each tester strain was tested in triplicate for the negative control group and in duplicate for the test substance-treated groups and the positive control groups.

Treatment of tester strains with the test substance and control solutions was conducted by the pre-incubation method. To 0.1 mL of the test substance or control solution, 0.5 mL of 0.1 mol/L sodium-phosphate buffer (pH 7.4) was added for the assays without metabolic activation, and 0.5 mL of S9 mix was used for the assays with metabolic activation. Culture medium (0.1 mL) was then added to the mixture and incubated in a shaking water bath (Personal-11 and THERMO MINDER EX, Taitec Corporation) at 37 °C for 20 min. Following pre-incubation, the mixture was added and mixed with 2 mL of top agar containing 0.05 mmol/L of L-histidine and 0.05 mmol/L D-biotin and spread over each minimum glucose agar plate. The top agar subsequently was solidified on a flat surface and the plates were incubated (MIR-262, SANYO Electronics Ltd.) at 37 °C for 49–50 h. Japanese Pharmacopoeia water (Otsuka Pharmaceutical Factory, Inc.) was used as the negative control substance and positive control mutagens included mitomycin C (MMC, Kyowa Hakko Kogyo Co., Ltd.), sodium azide (NaN_3, Wako Pure Chemical Industries, Ltd.), 9-aminoacridine hydrochloride hydrate

[1] Studies were conducted at the Safety Research Institute for Chemical Compounds Co. Ltd., (Sapporo, Japan) in compliance with Good Laboratory Practice standards as described under the "Ordinance on Standard of Conduct of Non-clinical Studies of Drug Safety" Ministry of Health and Welfare Ordinance No. 21 Japan, March 26, 1997 (genotoxicity studies), "Good Laboratory Practice Standards for Safety Studies on Drugs", Notification No. 313 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, March 31, 1982 (single dose oral toxicity study), or "Ordinance for Partial Amendment of the Ordinance on Standards for Conduct of Non-clinical Studies on the Safety of Drugs," Ministry of Health and Welfare Ordinance No. 114, Japan, June 13, 2008 (90-day repeat dose toxicity study).

[2] All animal investigations were conducted in accordance with all applicable laws and guidelines pertaining the humane treatment of animals as referenced below:"-Law for the Humane Treatment and Management of Animals," Law No. 105, October 1, 1973; Revised Law No. 221, December 22, 1999; Revised Law No. 68, June 22, 2005."Standards Relating to the Care, Management and Refinement of Laboratory Animals," Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006."Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in the Testing Facility under the Jurisdiction of the Ministry of Health, Labour and Welfare," Notification No. 0601005 of the Health Sciences Division, MHLW, Japan, June 1, 2006.

[3] Genotoxicity studies were conducted based "On Guidelines of Genotoxicity Study of Drugs," Notification No. 1604 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, November 1, 1999.

Table 1
Product specifications.

Parameter	Specification	
	AHCC-FD	AHCC-FG
Appearance	Brown powder	Light brown fine granules
Solubility	Highly soluble in water	Insoluble in water
Smell	Characteristic odor and taste	Sweet smell, bitter and sour taste
Taste		Bitter and sour taste
Particle size	–	99% pass 710 μm
Moisture (%)	NMT 5.0	NMT 3.0
Protein (%)	11 to 14	6 to 9
Lipid (%)	0.5 to 2.5	30 to 36
Carbohydrate (%)	74 to 81	49 to 55
AHCC-FD (%)		55 to 65
Ash (%)	6.5 to 8.5	3.5 to 5.5
Sodium (%)	0.8 to 1.5	3.5 to 5.5
Lead (ppm)	NMT 0.1	NMT 0.1
Arsenic (ppm)	NMT 1	NMT 1
Total bacteria (cfu/g)	NMT 1000 cfu/g	NMT 1000 cfu/g
Mold and Yeast	Not detected	Not detected
Coliforms	Negative	Negative
Staphylococcus	Not detected	Not detected
Salmonella	Negative	Negative

NMT, not more than; ppm, part per million.

(9-AA, Aldrich Chemical Co., Inc.), and 2-aminoanthracene (2-AA, Wako Pure Chemical Industries, Ltd.).

Each plate was observed for inhibition of cell growth using a stereoscopic microscope (SZ6045TR, Olympus Optical Co., Ltd.) and scored according to a Standard Operating Procedure. Plates also were checked for precipitation of the test substance. The number of revertant colonies was counted using a colony analyzer (CA-11D, System Science, Co., Ltd.).

In the second mutagenicity experiment, *S. typhimurium* tester strains TA98, TA100, and TA104 were used (obtained from National Institute of Hygienic Science, current National Institute of Health). In a dose-finding test, no increases in the number of revertant colonies or precipitation of the test substance in the assays without or with metabolic activation in any tester strain were observed. On the basis of these results, the highest concentration of the test substance in the main test was set at 5000 μg/plate, and a total of five concentrations (5000, 2500, 1250, 625, and 313 μg/plate) was used in both the assays without or with metabolic activation. The negative control was Japanese Pharmacopoeia water and the positive control mutagens included 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2, Wako Pure Chemical Industries, Ltd.) and 2-AA.

Cell growth inhibition was determined using a stereoscopic microscope. Precipitation of the test substance also was checked. Revertant colonies were counted using a colony counter (BMS-400, TOYO SOKKI CO., Ltd.). As for *S. typhimurium* TA98 whose revertant colonies are small in size, colony counting was conducted macroscopically using a digital colony counter (DC-3, Fuji Medical Co., Ltd.).

2.2.2. Micronucleus assay

AHCC-FD was dissolved in Japanese Pharmacopoeia purified water (Yakuhan Pharmaceutical Co., Ltd.) to prepare 50, 100, and 200 mg/mL solutions immediately before dosing, and was used within 1.4 h of preparation. MMC dissolved in purified water was used as the positive control, and Japanese Pharmacopoeia purified water was used as the negative control.

Thirty-four (34) specific pathogen free (SPF) Crlj:CD-1 (ICR) male mice aged 6 weeks (29.4–33.5 g) were obtained from Charles River Japan, Inc. Animals were quarantined and acclimatized for 6 days during which no abnormalities in general appearance were observed. The housing conditions were maintained at a temperature of 22 ± 3 °C, humidity of 50 ± 20%, ventilation of 10–15 air exchanges/hour, and lighting period of 12 h (artificial lighting from 8:00 to 20:00). Animals were accommodated in metallic bracket-type cages (260 W × 380 D × 180 H, mm) with wire mesh floors. Gamma-ray irradiated pellet diet (CRF-1, Oriental Yeast Co., Ltd.) was given *ad libitum* using metal feeders. The lot of the diet used was analyzed and inspected for potential chemical and bacterial contaminants that might affect the study.

Animals were assigned randomly to five test groups of six animals each according to body weight after excluding two animals with the lowest body weights and two with the highest body weights. Body weights at group assignment ranged from 34.0 to 37.5 g, which were within ±20% of the mean body weight.

Based on the results of the single dose oral toxicity study, the highest dose was set at 2000 mg/kg body weight, the upper limit dose specified in the guideline for genotoxicity studies on drugs, and a total of three doses were prepared using a series of twofold dilutions. The negative control group was treated with Japanese Pharmacopoeia purified water and a positive control group was treated with 1 mg/kg body weight of MMC. The test groups were set as shown in Table 2.

Administration of these substances was performed twice at an approximately 24-h interval using a syringe attached with a gastric tube. For the positive control substance, MMC, a single intraperitoneal dose was administered. Following dosing, animals were examined regularly for mortality and clinical signs of toxicity until sacrifice. Before each administration and between 18 and 24 h after the final administration, animals were weighed using an electronic balance (GX-2000, A & D Co., Ltd.).

Animals were euthanized by cervical dislocation 23–24 h after the final dose administration. Bone marrow specimens were prepared from five mice of each group. The bone marrow cells in the right and left femurs were flushed with fetal bovine serum and centrifuged (KR-702, KUBOTA Corporation) at 150*g* (1000 rpm) for 5 min to remove surplus serum. After removal of the serum and re-suspension of the cells, a drop of the cell suspension was smeared onto a slide glass. Each specimen was air-dried at room temperature overnight, and fixed with methanol (lot No. 801W1028, Kanto Chemical Co., Ltd.). Four specimens were prepared for each animal. After fixation with methanol, two specimens per animal were blind-coded. Each selected specimen was stained with 0.005% acridine orange stain (acridine orange, Wako Pure Chemical Industries, Ltd.), then washed with 1/15 mol/L phosphate buffer (pH 6.8, Mitsubishi Kagaku Iatron, Inc.), on which a cover glass was placed and sealed with enamel.

Specimens were observed with a fluorescence microscope (BX50: BX-FLA, Olympus Optical Co., Ltd.) at a total magnification of 1000×. Observations were conducted using 2000 polychromatic erythrocytes/animal (1000 polychromatic erythrocytes/specimen), and the percent of micronucleated polychromatic erythrocytes to total polychromatic erythrocytes (the incidence of micronuclei) was calculated. In addition, 500 erythrocytes/animal (250 erythrocytes/specimen) were observed, and the percent of polychromatic erythrocytes to total erythrocytes (the percent of polychromatic erythrocytes) was calculated. Group means and standard deviations of body weights were calculated. Body weights in the negative control group and the test substance groups were analyzed by the Bartlett test. The results showed homogeneity of variances ($p > 0.05$), so the data were analyzed by the one-way analysis of variance (ANOVA). ANOVA showed no significant difference, and subsequent analyses were not performed.

The incidences of micronuclei were compared between the negative control group and each test group by the conditional binominal test (Kastenbaum and Bowman, 1970). The test was conducted with upper-tailed significance levels of 5% and 1%. The data in the

Table 2
Experimental groups of the *in vivo* micronucleus assay.

Group/dosing substance	*N* (animal numbers)	Dose (mg/kg/d)	Dosing concentration (mg/mL)	Dosing volume (mL/kg/time)	Number of doses
Negative control (purified water)	6 (111 to 116)	–	–	10	2
AHCC-FD	6 (211 to 216)	500	50	10	2
	6 (311 to 316)	1000	100	10	2
	6 (411 to 416)	2000	200	10	2
Positive control (mitomycin C)	6 (511 to 516)	1	0.1	10	1

negative control group and those in each test group were analyzed by the *F* test (two-tailed) for homogeneity of variance between two groups. The results showed homogeneity of variances ($p > 0.10$), so Student's *t*-test (two-tailed) was performed for comparison between the two groups. These analyses were performed with significance levels of 5% and 1%.

2.3. Rodent toxicity studies[4]

2.3.1. Single dose oral toxicity study

Fifty-two (52) four-week-old male and 52 five-week-old female SPF Crj:CD Sprague–Dawley (SD) rats were received from Charles River Japan, Inc. These rats showing normal growth and no abnormalities following an acclimatization period of approximately one week were randomized by weight into two groups, each containing 10 males (127–135 g) and 10 females (134–142 g): a control group administered the vehicle (Japanese pharmacopoeia purified water) and a treatment group administered 12,500 mg/kg body weight AHCC-FD by gavage using a gastric tube. The dosing volume was 25 mL/kg body weight and the individual dosing volume was calculated based on body weight measurements obtained on the day of administration. A 500 mg/mL solution of AHCC-FD was prepared immediately prior to dosing. Dose confirmation tests indicated that the test article was 109.8% of the prescribed concentration; however, this deviation was unlikely to have adversely affected the results of the study. The animals were fasted for approximately 17 h prior to dosing and feed (CRF-1, Oriental Yeast Co., Ltd.) was provided *ad libitum* approximately 4 h after dose administration. The dose was selected based on the findings of a preliminary dose-finding study wherein no evidence of toxicity was observed in three male and three female rats treated with 12,500 mg/kg body weight of AHCC-FD, the highest possible dose.

All animals were observed immediately following dose administration, frequently during the first 6 h post-dosing, and once daily thereafter for a period of 14 days. Each animal was weighed on Day 0 (prior to dosing) and on Days 1, 3, 5, 7, 10, and 14. At the end of the observation period, all animals were euthanized under ether anesthesia and macroscopically observation conducted on all organs and tissues. The following organs and tissues were fixed and preserved in 10% neutral buffered formalin: liver, kidneys, spleen, heart, lung, brain, stomach, duodenum, jejunum, ileum, cecum, colon, and rectum.

2.3.2. 90-Day repeated dose toxicity study

2.3.2.1. Preparation method of the test substance. The required amount of AHCC-FD was accurately weighed and dissolved in purified water (Japanese pharmacopeia) to prepare 83.3, 250, and 500 mg/mL test solutions. The solutions were prepared once daily for the initial 13 days of the study and once or twice weekly thereafter. The test solutions, determined to be stable for a week at room temperature, were stored in a refrigerator (2–7 °C) in airtight bottles under light-resistance conditions.

2.3.2.2. Test animals. A total of 43 male (65–84 g) and 43 female (62–83 g), four-week-old SPF Slc:SD rats obtained from Japan SLC, Inc. were quarantined and acclimatized for a 5-day (males) or 6-day (females) period during which time the general appearance of the animals was monitored daily. Animals were distributed randomly to four groups of 10 male and 10 female rats per group according to body weight two days before the start of dosing. The rats were five weeks old at the beginning of the study period and body weights ranged from 130 to 156 g for the male and from 113 to 142 g for the female rats.

The animals were housed in bracket-type metallic cages (260 mm W × 380 mm D × 180 mm H) with wire mesh floors under regulated environmental conditions. During the acclimation period, two or three rats were housed per cage and following group assignment, animals were caged individually. Rats were provided free access to a pellet diet (CRF-1, Oriental Yeast Co., Ltd.) and tap water (city water of Sapporo-shi) supplied by an automatic watering system or water bottles. Dose levels of 1000, 3000, and 6000 mg/kg body weight/day administered by oral gavage were selected to represent, respectively, 33.3-, 100-, and 200-fold of the potential daily intake of AHCC-FD by humans of 1.8 g or 30 mg/kg body weight. A control group was treated with the vehicle only.

2.3.2.3. Test article administration. The test article was administered orally once daily, using a gastric tube, as an 83.3, 250, or 500 mg/mL solution of AHCC-FD. The dosing volume was 12 mL/kg body weight. The control group was dosed with an equivalent amount of purified water. The individual dosing volume was calculated based on body weight measurements obtained on the day nearest to the administration day.

2.3.2.4. Observation, measurement, and examination. Clinical observations: All animals were observed twice daily for general condition and any abnormal findings were recorded with times of onset and disappearance.

Body weight and food consumption: Body weight and food consumption measurements were measured twice weekly.

Ophthalmology: Ophthalmologic examinations were conducted on all animals prior to dose administration and on all control and high-dose group animals at the end of the study period.

Urinalysis: During week 13 of administration, animals were placed into metabolic cages for a period of 24 h for urine collection. Urine samples were analyzed for pH, protein, glucose, ketone body, urobilinogen, bilirubin, and occult blood using Multistix (Siemens Healthcare Diagnostics K.K). Urinary sediments were examined microscopically, urine volume was determined by a volumetric method, and specific gravity was measured by refractometry (Uricon-S, Atago Co. Ltd.).

Hematology and blood chemistry: Blood samples were collected for hematology and blood chemistry from all animals prior to scheduled necropsy at the end of the study period. All animals

[4] Single dose oral toxicity study was conducted in compliance with "On revision of Single-Dose and Repeated Dose-Toxicity studies" Notification No. 88 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, August 10, 1993. The 90-day toxicity study was conducted in compliance with the OECD Guidelines for the Testing of Chemicals "Repeated Dose 90-day Oral Toxicity Study in Rodents (408), 21st September 1998.

were fasted for 16–21 h prior to blood collection. Blood was collected from the abdominal aorta under ether anesthesia.

For hematology analysis, some of the blood was treated with EDTA-2K (VENOJECT®II, Terumo Corporation) to determine (Hematology analyzer F-820, Sysmex Co., Ltd.) the following parameters: red blood cell count, hematocrit, hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, platelet count, white blood cell count (WBC), and differential count of WBC. The reticulocyte count and differential count of WBC were determined by microscopy. A portion of the blood collected was treated with 3.8% sodium citrate and centrifuged at 3500 rpm for 10 min to obtain plasma, which was used to measure prothrombin time and activated partial thromboplastin time (Coagulator KC4Δ, Trinity Biotech Plc.).

For the determination (Hitachi 7080 automatic analyzer, Hitachi High-Technologies Co.) of blood chemistry parameters, blood samples were treated with approximately 20 units per mL of blood of heparin sodium (Ajinomoto Co., Inc.) and subsequently centrifuged at 3500 ppm for 10 min to obtain plasma. The following plasma parameters were measured: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase, and glucose. Serum samples were obtained by collecting blood samples in tubes containing separator (Sepaclean, Eiken Kizai Co., Ltd.) and subsequent centrifugation at 3500 ppm for 10 min and were used for the determination of total cholesterol, triglyceride, total bilirubin, urea nitrogen, creatinine, sodium, potassium, chloride, calcium, inorganic phosphorus, total protein, protein fraction, albumin/globulin ratio (A/G ratio). An AES320 automatic electrophoresis system (Mishima Olympus Co., Ltd.) was used to analyze protein fraction and A/G ratio.

Necropsy: At the end of the study, all animals were euthanized by exsanguination. The following organs and tissues were examined macroscopically and fixed and preserved in 10% neutral buffered formalin solution: liver, kidneys, spleen, heart, lung (including bronchus), brain (cerebrum, cerebellum, and pons cerebelli), pituitary, adrenals, thyroids, parathyroids, thymus, mesenteric lymph node, pancreas, tongue, mandibular lymph nodes, salivary glands (submandibular glands, sublingual glands), mammary gland (right abdominal region in principle, females only), skin (right abdominal region), eyeballs, harderian glands, sternum and right femur (including bone marrow), spinal cord (cervix), skeletal muscle (lateral vastus muscle), thoracic aorta, trachea, esophagus, stomach (fore stomach, glandular stomach, and pyrolic region), duodenum, jejunum, ileum (including Peyer's patches), cecum, colon, rectum, urinary bladder, testes, epididymides, prostate, seminal vesicle (including coagulating gland), ovaries, uterus (horn and cervix), vagina, peripheral nerve (sciatic nerve, right), and all gross lesions (with a border of normal tissue).

Organ weights: Absolute and relative organs weights were determined for the brain, pituitary, thymus, thyroids (including parathyroids), adrenals, spleen, heart, liver, kidneys, testes, epididymides and ovaries. Bilateral organs were measured together.

Histopathology: All organs and tissues fixed and preserved at necropsy from all animals in the control and high-dose group were examined microscopically. The samples were embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin. If any organs or tissues showed changes indicative of effect related to the test substance, preparations of all animals in the low- and mid-dose group also were examined microscopically. The organs and tissues showing gross abnormalities were examined microscopically.

2.3.3. Statistical analyses

For quantitative parameters, averages and standard deviations were calculated, and analyzed by the Bartlett test for homogeneity of variances. If there was evidence of homogeneity ($p > 0.05$), ANOVA was used, and significant differences ($p \leqslant 0.10$) were analyzed by Dunnett's multiple comparison test to compare with the control group. If there was no evidence of homogeneity ($p \leqslant 0.05$), the Kruskal–Wallis test was used, and significant differences ($p \leqslant 0.10$) were analyzed by the Mann–Whitney *U*-test to compare the AHCC-FD treated groups to the control group. Urinalysis parameters were analyzed by the Kruskal–Wallis test, and significant differences ($p \leqslant 0.10$) were analyzed by the Mann–Whitney *U*-test with the level of statistical significance set at 5%.

3. Results

3.1. Genotoxicity studies

3.1.1. Ames test

In both the Ames tests, the presence of AHCC-FD at doses of ranging from 156 to 5000 μg/plate did not increase the number of revertants per plate in any of the *S. typhimurium* strains tested, either in the presence or absence of metabolic activation (Table 3). As expected the positive control treatments produced an average number of revertant colonies that was over twice that of the negative control group. Precipitation of the test substance or inhibition of cell growth was not observed in either experiment.

On the basis of the above results, it was concluded that AHCC-FD was not mutagenic to the tester strains under the conditions of these tests.

3.1.2. In vivo mouse micronucleus study

No abnormal signs were observed in general appearance in the negative control group or any of the 500, 1000, or 2000 mg/kg body weight/day AHCC-FD test substance groups. Mean body weights measured before administration, at 24 h post-dosing, and between 18 and 24 h after the final administration in the 500, 1000, and 2000 mg/kg body weight/day test substance groups were comparable to those in the negative control group, and there were no statistically significant differences. In the positive control group, no abnormal signs were observed in general appearance after administration, and the mean body weight between 18 and 24 h after the final administration was comparable to the pre-administration value.

Table 4 shows the incidence of micronuclei and the percent of polychromatic erythrocytes. The incidence of micronuclei in the negative control and AHCC-FD treatment groups were not significantly different. The incidence of micronuclei in the positive control group was statistically increased when compared to the negative control group. The percent of polychromatic erythrocytes in the AHCC-FD treatment and positive control groups were not statistically different in comparison to the negative control group.

The results indicate that AHCC-FD was not genotoxic and was not toxic to blood forming cells.

3.2. Rodent toxicity studies

3.2.1. Single dose oral toxicity study

No deaths occurred in males or females in the control or 12,500 mg/kg groups. All the animals in the 12,500 mg/kg group showed a decrease in spontaneous activity shortly after AHCC-FD administration, which was accompanied with increase in water-intake activity or diarrhea. Brown urine was observed in one AHCC-FD-treated female (animal No. 260) from 30 min to two hours after the administration. In many of these animals, these signs disappeared by the termination of observation on the administration day.

Table 3
Results of Ames reverse mutation experiments conducted with AHCC-FD in the absence (−S9) and presence of metabolic activation (+S9).

Metabolic activation	Concentration (μg/plate)	Number of revertant colonies per plate (number in parentheses represent the mean number of colonies)											
		TA102		TA1535		TA1537		TA100		TA104		TA98	
−S9	0 (negative control)	384		7		8		134		442		17	
		324		7		6		122		498		22	
		333	(347)	11	(8)	7	(7)	114	(123)	527	(489)	20	(20)
	156	383		11		8		–		–		–	
		386	(385)	10	(11)	7	(8)	–		–		–	
	313	403		7		5		118		118		15	
		379	(391)	7	(7)	7	(6)	112	(115)	588	(576)	9	(12)
	625	377		8		7		109		513		19	
		403	(390)	6	(7)	8	(8)	108	(109)	527	(520)	16	(18)
	1250	422		9		6		118		482		15	
		411	(417)	10	(10)	5	(6)	115	(117)	535	(509)	16	(16)
	2500	411		8		5		116		534		20	
		450	(431)	5	(7)	7	(6)	103	(110)	547	(541)	17	(19)
	5000	461		12		11		136		550		15	
		398	(430)	7	(10)	7	(9)	130	(133)	656	(603)	24	(20)
+S9	0 (negative control)	364		7		6		141		566		34	
		402		7		10		140		605		35	
		384	(383)	9	(8)	10	(9)	130	(137)	554	(575)	40	(36)
	156	395		13		8		–		–		–	
		402	(399)	7	(10)	12	(10)	–		–		–	
	313	424		7		7		150		556		29	
		392	(408)	11	(9)	8	(8)	140	(145)	451	(504)	25	(27)
	625	391		11		8		158		475		27	
		389	(390)	8	(10)	10	(9)	151	(155)	567	(521)	32	(30)
	1250	407		12		11		144		581		35	
		390	(399)	7	(10)	10	(11)	136	(140)	522	(552)	31	(33)
	2500	400		9		7		157		611		35	
		412	(406)	6	(8)	6	(7)	136	(147)	697	(654)	28	(32)
	5000	438		5		10		182		600		31	
		415	(427)	8	(7)	12	(11)	145	(164)	548	(574)	38	(35)
Positive Controls													
−S9	Positive control	MMC		NaN_3		9-AA		AF-2		AF-2		AF-2	
	Concentration (μg/plate)	0.05		0.5		80		0.01		0.01		0.1	
	No. of revertant colonies/plate	1297		200		194		688		1309		484	
		1343	(1320)	262	(231)	289	(242)	625	(657)	1283	(1296)	470	(477)
+S9	Positive control	2-AA		2-AA		2-AA		2-AA		2-AA		2-AA	
	Concentration (μg/plate)	5		2		2		1		5		0.5	
	No. of revertant colonies/plate	1518		322		182		1387		3072		299	
		1437	(1478)	310	(316)	197	(190)	1333	(1360)	2931	(3002)	344	(322)

2-AA, 2-aminoanthracene; 9-AA, 9-aminoacridine hydrochloride hydrate; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; MMC, mitomycin C; NaN_3, sodium azide.

Table 4
Results of the micronucleus assay with AHCC-FD in mice.

Group	Dose[a] (mg/kg/day)	Animal number	% MNPCE[b]	% PCE[c]
Negative control (purified water)	–	111	0.10	41.2
		112	0.20	38.0
		113	0.05	42.4
		114	0.00	38.0
		115	0.05	49.2
		Mean ± SD	0.08 ± 0.08	41.8 ± 4.6
AHCC-FD	500	211	0.15	39.4
		212	0.00	45.8
		213	0.10	40.2
		214	0.25	38.8
		215	0.20	47.8
		Mean ± SD	0.14 ± 0.10	42.4 ± 4.1
	1000	311	0.15	53.0
		312	0.00	39.8
		313	0.25	44.6
		314	0.20	54.6
		315	0.15	42.8
		Mean ± SD	0.15 ± 0.09	47.0 ± 6.5
	2000	411	0.30	39.4
		412	0.05	36.0
		413	0.15	58.8
		414	0.05	50.2
		415	0.20	52.4
		Mean ± SD	0.15 ± 0.11	47.4 ± 9.4
Positive control (mitomycin C)	1 mg/kg	511	4.80	52.4
		512	2.85	29.6
		513	2.45	34.8
		514	4.15	27.0
		515	3.50	41.0
		Mean ± SD	3.55 ± 0.95[d]	37.0 ± 10.2

MNPCE, micronucleated polychromatic erythrocytes; PCE, polychromatic erythrocytes.
NMT, not more than; ppm, part per million.
[a] Two successive oral doses (24 h apart) except mitomycin C (single intraperitoneal injection).
[b] % of MNCPE based on 2000 PCEs per animal.
[c] % of PCE based on 500 erythrocytes per animal.
[d] Statistically significant difference from the control ($p \leqslant 0.01$, conditional binomial test).

Table 5 shows the body weights of the rats at Day 0 (before administration) and at Days 7, 10, and 14 following administration. Body weight gain tended to be lower throughout the observation period for male rats administered AHCC-FD relative to controls, and statistically significant differences were observed on Days 10 and 14. No statistically significant differences in body weight gain were observed in female rats. At necropsy, one female (animal No. 255) in the 12,500 mg/kg group showed dilatation of renal pelvis.

3.2.2. Repeat dose oral toxicity study

Clinical observations: All control and AHCC-FD-treated animals survived the 90-day study period. No abnormal signs or symptoms attributed to the test substance were observed. Partial white opacity in the right eye was observed in one male of the low-dose (1000 mg/kg body weight) group on and after Day 13 and trauma in the third digit of the forelimb was observed in a high-dose (6000 mg/kg body weight) female on and after Day 87. These findings were considered incidental and of no toxicological significance.

Body weight and food consumption: There was no significant difference in body weight gain among the control and AHCC-FD treated groups (Figs. 1 and 2).

No meaningful differences in food consumption were observed between the treatment and control groups. Relative to the control group, significantly increased food intake was observed on Days 22, 25, 32, and 53 of administration for females of the low-dose group and, on Day 67, food intake in females of the high-dose group was significantly reduced. These transient changes were neither dose-dependent nor accompanied by significant body weight changes and were considered of no biological significances.

Ophthalmology: There were no abnormal ophthalmological findings at baseline or after 13 weeks of treatment.

Urinalysis: Urinalysis did not reveal any statistically significant changes in the low-dose group. Decreased pH values in mid- and high-dose females and in high-dose males were statistically significant ($p \leqslant 0.01$, Mann–Whitney's *U*-test) (Table 6). A greater degree of protein excretion was observed in high-dose animals of both sexes ($p \leqslant 0.01$) and in female rats of the mid-dose ($p \leqslant 0.05$). For male rats in the high-dose groups, specific gravity values were elevated significantly relative to the control group ($p \leqslant 0.01$). Analyses of urinary sediment did not reveal any meaningful differences among control and treatment groups (data not shown).

Hematology and blood chemistry: No statistically significant differences were observed in any of the hematological parameters among males and females of the AHCC-FD-treated and control groups (Table 7). Blood chemistry results are summarized in Table 8. Serum total protein concentration was significantly higher in the low-dose group females; however, this change was not dose-dependent and was not observed in male rats. In the high-dose group, slight, but statistically significant, increases in serum levels of γ-globulin and creatinine in female and male rats, respectively, were considered incidental as corresponding changes attributable to AHCC-FD treatment were not detected in the kidneys.

Serum triglycerides were significantly elevated for all AHCC-FD-treated females (18.0, 46.4, 27.6, 26.2 mg/dL for control, low-, mid-, and high-dose groups, respectively); however, no clear dose–response relationship was evident.

Necropsy: No macroscopic findings related to treatment with AHCC-FD were detected in the low- and mid-dose groups. In the high-dose group, a black patch in the mucosa of the glandular stomach was observed in one female rat. Adhesion between lateral left lobe and papillary process of caudal lobe of the liver also was observed in the same female rat of the high-dose group.

The following observations at necropsy were not considered treatment related: a white mass in the limiting ridge of the stomach observed in one male rat of each control and high-dose group; focal white opacity in the right eyeball of a male in the low-dose group; diverticulum in the ileum in a female of the mid-dose group; and trauma in the third digit of the left forelimb observed in a high-dosed female.

Organ weights: The absolute and relative organs weights are summarized in Table 9. The only statistically significant differences when compared to control group were reduced relative brain weights for females of the low-dose group and decreased relative adrenal weight for males of the mid-dose group. These changes were not dose-dependent and were considered to be of no toxicological significance.

Histopathology: In the high-dose group, slight squamous cell hyperplasia of the limiting ridge of the stomach was observed in all 10 males and three females, which was accompanied by slight hyperkeratosis in three males and in the same three female rats (Table 10). No changes in the limiting ridge of the stomach were detected in any animals of the low- and mid-dose groups. In the glandular stomach, ectopic glandular tissue in the submucosa was observed in a male and slight erosion was observed in a female of the high-dose group. In the liver, slight fatty changes were observed in the centrilobular hepatocytes of five high-dose group males and in the periportal hepatocytes of one low-dose group female rat.

Table 5
Body weights of male and female rats following single dose administration of AHCC-FD.

Animal No.	Control group				Animal No.	12,500 mg/kg body weight			
	Day 0	Day 7	Day 10	Day 14		Day 0	Day 7	Day 10	Day 14
Male animals									
101	108	192	222	258	201	109	192	220	259
102	109	205	238	282	202	109	185	214	252
103	109	190	221	255	203	110	185	212	244
104	111	195	220	260	204	110	198	228	272
105	110	196	230	267	205	109	203	229	259
106	112	200	232	269	206	108	186	218	256
107	109	205	236	274	207	111	198	220	257
108	108	196	222	257	208	108	191	216	255
109	110	189	219	256	209	116	197	222	257
110	112	193	224	259	210	103	174	197	233
Mean	109.8	196.1	226.4	263.7	Mean	109.3	190.9	217.6[a]	254.4[a]
SD	1.5	5.7	7.0	9.0	SD	3.2	8.6	9.1	10.2
SE	0.5	1.8	2.2	2.8	SE	1.0	2.7	2.9	3.2
Female animals									
151	115	172	181	198	251	118	161	173	188
152	117	166	170	181	252	115	167	181	201
153	116	162	174	188	253	113	157	177	195
154	118	176	182	200	254	116	171	200	218
155	116	170	182	194	255	119	177	193	211
156	120	162	178	193	256	120	166	172	178
157	120	173	181	201	257	119	176	183	197
158	119	167	179	196	258	118	175	181	200
159	119	181	192	213	259	119	178	189	200
160	128	176	186	200	260	119	173	184	201
Mean	118.8	170.5	180.5	196.4	Mean	117.6	170.1	183.3	198.9
SD	3.7	6.3	6.0	8.5	SD	2.2	7.1	8.8	11.0
SE	1.2	2.0	1.9	2.7	SE	0.7	2.3	2.8	3.5

[a] Statistically significant difference from the control ($p \leqslant 0.05$, Dunnett's procedure).

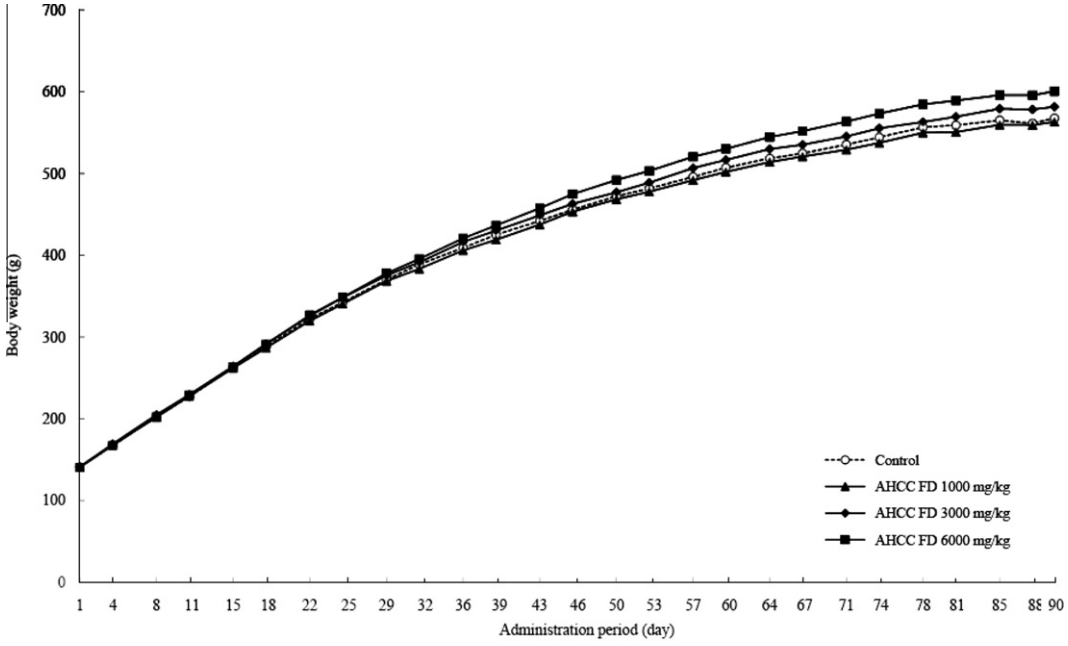

Fig. 1. Body weight changes in male rats treated orally with AHCC for 90 days.

4. Discussion

There is mounting interest in fungal derived glucans due to their purported immunostimulatory effects. AHCC has been used in Japan for more than a decade as a dietary supplement to boost immune function as well as in clinical trials as an adjunct in the treatment in hepatocellular carcinoma (Matsui et al., 2002; Spierings et al., 2007). AHCC also is widely used in Japan to improve the general feeling of well-being in cancer and liver disease patients (Spierings et al., 2007). Although, some physicians recommended up to 6 g/day, the typical daily recommended dose of AHCC-FG in clinical practice is 3 g (equivalent to 1.8 g AHCC-FD). A limited number of studies involving AHCC administration in healthy subjects have been published (Spierings et al., 2007;

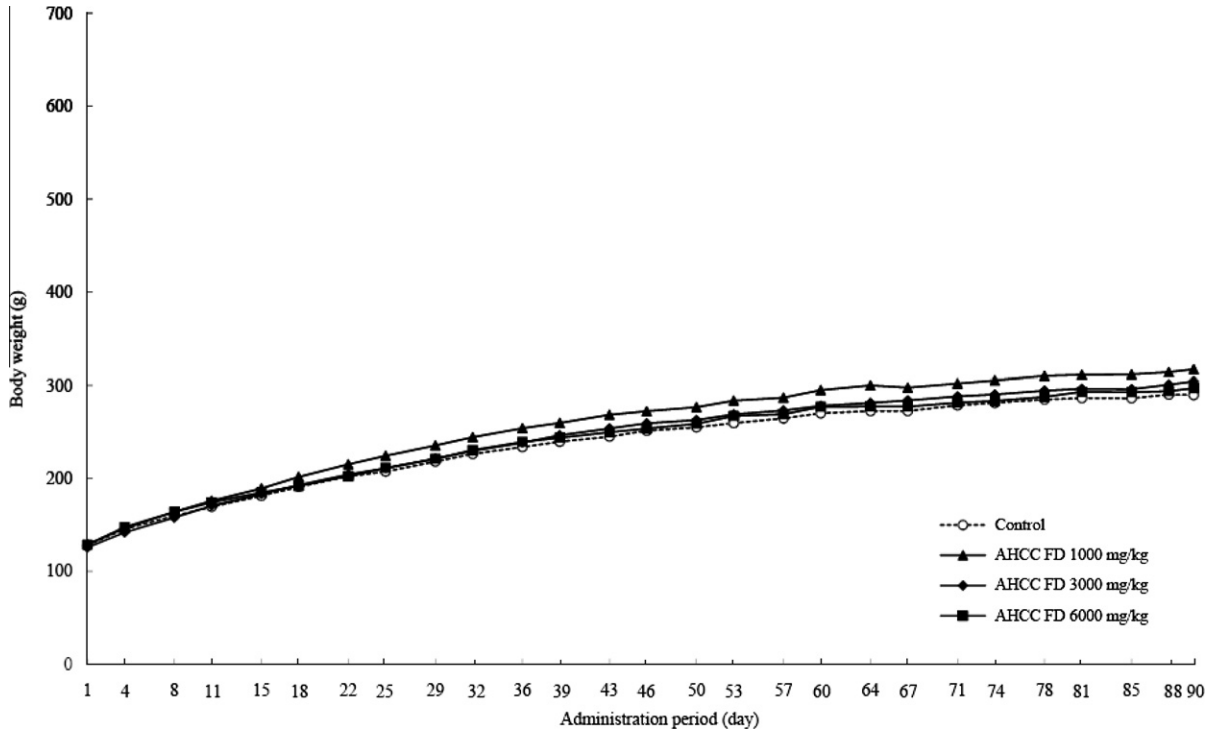

Fig. 2. Body weight changes in female rats treated orally with AHCC for 90 days.

Table 6
Urinalysis results for of male and female rats following 90-day gavage administration of AHCC-FD.

Parameters measured		Male				Female			
Dose (mg/kg body weight/day)		0	1000	3000	6000	0	1000	3000	6000
Number of animals examined		10	10	10	10	10	10	10	10
pH	6.0	0	0	0	2	0	0	1	3
	6.5	0	0	2	3	0	0	3	6
	7.0	0	0	2	4	0	0	1	1
	7.5	1	1	1	0	0	3	2	0
	8.0	3	3	2	0	1	2	1	0
	8.5	6	6	3	1++	9	5	2++	0++
Protein	–	1	0	0	0	3	0	0	0
	±	1	3	0	0	5	7	4	2
	1+	8	6	6	3	2	2	4	7
	2+	0	1	4	6	0	1	2	1
	3+	0	0	0	1++	0	0	0+	0++
Glucose	–	10	10	10	10	10	10	10	10
Ketone body	–	10	10	10	10	10	10	10	10
Urobilinogen	0.1 EU/dL	10	10	10	10	10	10	10	10
Bilirubin	–	10	10	10	10	10	10	10	10
Occult blood	–	4	4	8	7	9	7	9	8
	±	3	4	2	3	0	3	1	2
	1+	1	1	0	0	0	0	0	0
	2+	2	0	0	0	1	0	0	0
	3+	0	1	0	0	0	0	0	0
Specific gravity	1.000–1.010	0	0	0	0	0	1	0	0
	1.011–1.020	0	0	0	0	0	0	0	1
	1.021–1.030	1	0	0	0	2	2	2	0
	1.031–1.040	7	5	3	1	4	3	3	4
	1.041–1.050	1	4	4	5	2	4	3	4
	1.050<	1	1	3	4++	2	0	2	1
Urine volume	mL/21 h	17.05 ± 4.05	14.55 ± 2.15	14.40 ± 3.91	14.90 ± 3.44	10.35 ± 3.46	15.75 ± 13.21	12.20 ± 6.33	13.50 ± 8.82

Values are number of animals with findings.
–, normal; ±, slight; 1+, moderate; 2+, severe; 3+, very severe.
+., significantly different from the control groups ($p \leqslant 0.05$, Mann–Whitney's *U*-test).
++, significantly different from the control groups ($p \leqslant 0.01$, Mann–Whitney's *U*-test).

Table 7
Hematology results for male and female rats following daily gavage administration of AHCC-FD for a period of 90 days.

Parameter (units)	Dose group (mg/kg body weight/day)							
	Male (n = 10)				Female (n = 10)			
	0	1000	3000	6000	0	1000	3000	6000
RBC ($\times 10^4/\mu L$)	927.7 ± 32.5	924.4 ± 13.6	930.4 ± 25.6	910.7 ± 30.6	888.5 ± 26.0	861.5 ± 21.6	883.4 ± 32.3	864.1 ± 40.0
Ht (%)	46.71 ± 1.07	46.54 ± 1.17	46.82 ± 1.02	46.36 ± 1.44	47.36 ± 1.00	46.15 ± 1.25	46.75 ± 1.86	45.94 ± 1.79
Hb (g/dL)	15.72 ± 0.27	15.58 ± 0.43	15.66 ± 0.38	15.45 ± 0.54	16.39 ± 0.32	15.95 ± 0.51	16.12 ± 0.60	15.88 ± 0.59
MCV (fL)	50.38 ± 1.33	50.37 ± 1.32	50.33 ± 0.80	50.94 ± 1.61	53.33 ± 0.74	53.60 ± 1.23	52.93 ± 1.03	53.19 ± 0.92
MCH (pg)	16.96 ± 0.48	16.85 ± 0.43	16.85 ± 0.46	16.97 ± 0.54	18.47 ± 0.29	18.50 ± 0.49	18.25 ± 0.34	18.39 ± 0.43
MCHC (g/dL)	33.65 ± 0.27	33.49 ± 0.30	33.47 ± 0.54	33.34 ± 0.29	34.62 ± 0.36	34.56 ± 0.35	34.49 ± 0.21	34.57 ± 0.49
WBC ($\times 10^2/\mu L$)	87.6 ± 16.7	87.2 ± 11.9	85.0 ± 11.4	95.7 ± 16.2	53.0 ± 10.0	56.9 ± 12.8	53.8 ± 11.2	60.8 ± 8.0
Platelet count ($10^4/\mu L$)	95.69 ± 10.51	104.59 ± 9.37	102.11 ± 7.26	103.65 ± 11.11	103.24 ± 11.35	104.13 ± 9.37	104.98 ± 3.67	105.95 ± 11.26
Reticulocyte count (%)	28.0 ± 3.1	28.3 ± 2.4	25.7 ± 1.8	26.1 ± 2.8	24.4 ± 2.8	23.7 ± 4.6	22.0 ± 4.3	22.5 ± 2.5
PT (s)	17.01 ± 1.22	16.63 ± 1.01	16.83 ± 1.17	16.33 ± 0.67	16.57 ± 0.54	16.35 ± 0.39	16.79 ± 0.55	17.08 ± 0.43
APTT (s)	25.98 ± 2.11	24.79 ± 1.61	24.91 ± 2.07	24.56 ± 1.83	20.06 ± 1.12	20.02 ± 1.53	20.07 ± 1.21	19.57 ± 0.87
Differential WBC counts (%)								
Neutrophil (stab form)	1.60 ± 0.98	1.24 ± 0.72	2.04 ± 0.55	1.08 ± 0.91	1.16 ± 0.30	1.36 ± 0.43	1.12 ± 0.77	1.32 ± 0.68
Neutrophil (segmented)	15.00 ± 6.08	13.60 ± 3.35	16.12 ± 5.56	13.64 ± 4.02	17.52 ± 3.73	17.48 ± 5.72	18.52 ± 5.57	14.44 ± 3.03
Eosinophil	1.48 ± 0.71	2.00 ± 1.80	2.00 ± 1.08	0.96 ± 0.63	2.36 ± 1.12	1.72 ± 0.73	2.20 ± 1.51	1.24 ± 1.26
Basophil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Monocyte	2.36 ± 0.97	2.40 ± 0.88	2.56 ± 1.18	2.12 ± 1.13	1.88 ± 0.96	2.56 ± 1.38	2.12 ± 1.03	1.24 ± 0.89
Lymphocyte	79.56 ± 7.33	80.76 ± 5.34	77.28 ± 6.70	82.20 ± 4.46	77.08 ± 3.79	76.88 ± 5.79	76.04 ± 6.68	81.76 ± 3.25
Others	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

All values represent the mean ± SD.
APTT, activated partial thromboplastin time; Hb, hemoglobin; Ht, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PT, prothrombin time; RBC, red blood cell count; WBC, white blood cell count.

Table 8
Blood chemistry results for male and female rats following daily gavage administration of AHCC-FD for a period of 90 days.

Parameters (units)	Dose group (mg/kg body weight/day)							
	Male (n = 10)				Female (n = 10)			
	0	1000	3000	6000	0	1000	3000	6000
Total protein (g/dL)	6.19 ± 0.25	6.17 ± 0.09	6.33 ± 0.24	6.28 ± 0.27	6.26 ± 0.27	6.61 ± 0.29[a]	6.57 ± 0.28	6.37 ± 0.36
A/G ratio	0.955 ± 0.046	0.975 ± 0.058	0.905 ± 0.048	0.920 ± 0.055	1.179 ± 0.074	1.192 ± 0.078	1.219 ± 0.083	1.139 ± 0.098
Protein fraction (%)								
Albumin	48.78 ± 1.24	49.34 ± 1.47	47.54 ± 1.39	47.88 ± 1.46	54.04 ± 1.57	54.32 ± 1.65	54.88 ± 1.71	53.17 ± 2.22
α1-globulin	21.80 ± 1.12	22.58 ± 2.04	22.92 ± 1.38	23.3 ± 1.22	15.80 ± 1.92	16.80 ± 1.09	16.29 ± 2.15	16.41 ± 1.28
α2-globulin	8.44 ± 1.04	8.10 ± 1.05	8.12 ± 1.22	8.18 ± 1.03	9.02 ± 0.72	8.47 ± 0.48	8.43 ± 0.80	8.81 ± 0.60
β-globulin	16.58 ± 0.85	15.99 ± 0.56	16.61 ± 0.84	16.50 ± 0.27	16.40 ± 0.56	15.55 ± 1.31	15.65 ± 0.70	15.98 ± 0.87
γ-globulin	4.40 ± 0.87	3.99 ± 1.00	4.81 ± 1.09	4.14 ± 0.76	4.74 ± 0.39	4.86 ± 0.54	4.75 ± 1.07	5.63 ± 0.98+
AST (IU/L)	69.1 ± 12.1	68.5 ± 14.0	69.7 ± 9.8	74.8 ± 17.2	73.0 ± 24.2	80.4 ± 23.9	73.2 ± 17.1	76 ± 28.9
ALT (IU/L)	40.7 ± 7.2	41.1 ± 12.3	41.0 ± 8.1	41.5 ± 5.2	37.5 ± 13.3	47.2 ± 19.8	44.6 ± 10.4	44.4 ± 17.9
ALP (IU/L)	389.9 ± 99.6	354.5 ± 38.9	351.0 ± 58.8	361.9 ± 50.6	209.6 ± 30.4	212.7 ± 42.9	210.2 ± 33.7	233.8 ± 52.4
γ-GTP, IU/L)	0.39 ± 0.18	0.38 ± 0.15	0.43 ± 0.21	0.30 ± 0.14	1.08 ± 0.43	1.03 ± 0.31	1.13 ± 0.54	1.41 ± 0.69
T-Bil (mg/dL)	0.047 ± 0.011	0.051 ± 0.009	0.053 ± 0.011	0.053 ± 0.008	0.048 ± 0.009	0.044 ± 0.011	0.046 ± 0.015	0.046 ± 0.011
Glucose (mg/dL)	168.7 ± 8.4	174.7 ± 19.6	176.5 ± 6.2	177.3 ± 9.7	159.7 ± 13.5	159.4 ± 10.7	157.1 ± 7.8	153.4 ± 8.3
T-Cho (mg/dL)	73.5 ± 11.0	68.1 ± 11.1	69.1 ± 11.8	69.2 ± 14.7	94.4 ± 14.0	95.0 ± 10.9	89.2 ± 13.1	89.0 ± 11.2
TG (mg/dL)	136.5 ± 48.5	120.1 ± 26.0	105.0 ± 34.9	134.2 ± 56.3	18.0 ± 2.9	46.4 ± 17.6++	27.6 ± 10.6+	26.2 ± 10.0++
UN (mg/dL)	14.69 ± 1.42	14.68 ± 1.06	14.23 ± 1.21	13.92 ± 1.17	17.11 ± 1.59	16.69 ± 1.49	16.80 ± 2.24	17.28 ± 1.48
Creatinine (mg/dL)	0.551 ± 0.020	0.576 ± 0.038	0.563 ± 0.028	0.584 ± 0.031[a]	0.606 ± 0.060	0.607 ± 0.047	0.582 ± 0.048	0.602 ± 0.047
Sodium (mEq/L)	140.8 ± 0.4	141.5 ± 0.8	140.8 ± 0.9	140.9 ± 1.1	140.5 ± 1.2	140.2 ± 1.4	139.9 ± 1.4	139.8 ± 0.6
Potassium (mEq/L)	4.925 ± 0.219	4.921 ± 0.166	4.911 ± 0.289	4.880 ± 0.190	4.784 ± 0.334	4.628 ± 0.319	4.849 ± 0.143	4.843 ± 0.157
Chloride (mEq/L)	104.3 ± 1.3	104.5 ± 1.1	103.8 ± 1.4	103.2 ± 1.4	106.7 ± 1.8	105.7 ± 2.1	105.2 ± 1.8	105.9 ± 1.7
Calcium (mg/dL)	10.18 ± 0.23	10.09 ± 0.25	10.01 ± 0.29	10.15 ± 0.28	9.86 ± 0.19	10.03 ± 0.28	10.10 ± 0.18	9.77 ± 0.29
IP (mg/dL)	6.02 ± 0.80	6.13 ± 0.59	5.85 ± 0.73	5.82 ± 0.57	4.78 ± 0.78	4.58 ± 0.84	4.62 ± 0.64	4.21 ± 0.82

All values represent the mean ± SD.
A/G ratio, albumin/globulin ratio; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; IP, inorganic phosphorus; T-Bil, total bilirubin; T-Cho, total cholesterol; TG, triglyceride; UN, urea nitrogen.
+, significantly different from the control group at $p \leqslant 0.05$ (Mann–Whitney's U-test).
++, significantly different from the control group at $p \leqslant 0.01$ (Mann–Whitney's U-test).
[a] Significantly different from the control group at $p \leqslant 0.05$ (Dunnett's test).

Terakawa et al., 2008). The safety and tolerability of AHCC in humans were evaluated by Spierings and colleagues (2007) in a group of 26 healthy adults. The study was conducted in accordance with the United States Food and Drug Administration guidelines for Phase I trials and employed an open label non-controlled intervention study design. The test article administered during the study was formulated as an AHCC-FD containing drink (Amino Up Chemical Co., Ltd., personal communication) with subjects consuming up to 9 g of AHCC-FG daily for a period of 14 days, a dose equivalent to 2.5–5 times greater than the dose recommended in clinical applications. Relative to baseline values, no significant changes in laboratory parameters, including ALT, AST,

Table 9
Absolute and relative organ weights for male and female following daily gavage administration of AHCC-FD for a period of 90 days.

Organ	Dose group (mg/kg body weight/day)							
	Male (n = 10)				Female (n = 10)			
	0	1000	3000	6000	0	1000	3000	6000
Absolute organ weights (g, unless stated otherwise)								
Body weight	534.3 ± 38.2	532.5 ± 43.1	550.4 ± 75.6	569.4 ± 67.4	272.1 ± 17.3	295.7 ± 30.7	282.2 ± 24.4	280.1 ± 22.3
Liver	14.388 ± 1.274	14.826 ± 1.742	14.810 ± 2.711	16.352 ± 2.767	6.538 ± 0.341	7.358 ± 1.097	6.966 ± 0.752	7.095 ± 0.717
Kidneys	2.896 ± 0.219	2.903 ± 0.247	3.087 ± 0.488	3.228 ± 0.410	1.609 ± 0.113	1.728 ± 0.183	1.739 ± 0.160	1.77 ± 0.206
Spleen	0.891 ± 0.125	0.889 ± 0.100	0.846 ± 0.113	0.894 ± 0.116	0.514 ± 0.075	0.54 ± 0.058	0.520 ± 0.084	0.568 ± 0.069
Heart	1.434 ± 0.121	1.414 ± 0.127	1.436 ± 0.160	1.504 ± 0.129	0.868 ± 0.078	0.873 ± 0.075	0.898 ± 0.037	0.879 ± 0.079
Brain	2.221 ± 0.078	2.199 ± 0.048	2.233 ± 0.070	2.227 ± 0.066	2.037 ± 0.071	2.003 ± 0.058	2.083 ± 0.069	2.044 ± 0.069
Pituitary (mg)	11.86 ± 0.79	12.26 ± 1.27	12.21 ± 2.28	12.62 ± 0.93	15.72 ± 2.44	16.92 ± 4.37	13.93 ± 2.82	15.00 ± 1.90
Thymus (mg)	416.7 ± 51.7	407.7 ± 79.4	432.2 ± 77.8	413.9 ± 92.5	319.3 ± 48.2	366.0 ± 52.1	353.0 ± 68.3	322.9 ± 38.6
Thyroids (mg)	18.32 ± 4.23	19.21 ± 3.51	19.76 ± 4.41	21.03 ± 3.75	13.56 ± 3.49	14.12 ± 3.58	12.89 ± 1.89	12.09 ± 2.25
Adrenals (mg)	71.7 ± 7.6	72.3 ± 6.1	64.0 ± 15.1	70.8 ± 12.4	79.7 ± 8.4	79.9 ± 9.3	77.3 ± 9.7	77.8 ± 13.9
Testes	3.953 ± 0.269	3.907 ± 0.175	3.855 ± 0.295	3.824 ± 0.350	–	–	–	–
Epididymides	1.304 ± 0.084	1.305 ± 0.063	1.34 ± 0.084	1.290 ± 0.083	–	–	–	–
Ovaries (mg)	–	–	–	–	77.1 ± 6.4	80.4 ± 13.1	80.0 ± 15.1	84.8 ± 8.9
Relative organ weights (% of bw, unless stated otherwise)								
Liver	2.689 ± 0.139	2.781 ± 0.204	2.701 ± 0.346	2.859 ± 0.163	2.409 ± 0.128	2.482 ± 0.193	2.467 ± 0.127	2.532 ± 0.135
Kidneys	0.543 ± 0.032	0.547 ± 0.036	0.560 ± 0.028	0.566 ± 0.028	0.592 ± 0.034	0.583 ± 0.044	0.618 ± 0.056	0.632 ± 0.040
Spleen	0.168 ± 0.013	0.168 ± 0.013	0.155 ± 0.019	0.159 ± 0.019	0.190 ± 0.021	0.185 ± 0.013	0.185 ± 0.022	0.203 ± 0.026
Heart	0.267 ± 0.016	0.267 ± 0.016	0.263 ± 0.020	0.266 ± 0.020	0.32 ± 0.023	0.298 ± 0.015	0.320 ± 0.026	0.314 ± 0.016
Brain	0.417 ± 0.025	0.414 ± 0.028	0.412 ± 0.059	0.394 ± 0.037	0.751 ± 0.054	0.685 ± 0.067[a]	0.744 ± 0.075	0.733 ± 0.037
Pituitary (10–3%)	2.227 ± 0.177	2.307 ± 0.221	2.217 ± 0.296	2.228 ± 0.136	5.806 ± 1.047	5.675 ± 1.046	4.925 ± 0.859	5.371 ± 0.694
Thymus (10^{-3}%)	78.500 ± 12.349	76.645 ± 13.562	79.014 ± 13.969	72.663 ± 14.579	117.219 ± 14.834	123.909 ± 12.664	124.954 ± 21.453	115.555 ± 13.465
Thyroids (10^{-3}%)	3.418 ± 0.682	3.607 ± 0.571	3.596 ± 0.664	3.681 ± 0.346	4.955 ± 1.080	4.805 ± 1.226	4.605 ± 0.804	4.320 ± 0.790
Adrenals (10^{-3}%)	13.455 ± 1.477	13.590 ± 0.666	11.654 ± 2.368+	12.426 ± 1.371	29.327 ± 2.773	27.184 ± 3.424	27.563 ± 3.899	27.684 ± 3.706
Testes	0.741 ± 0.047	0.736 ± 0.053	0.709 ± 0.071	0.677 ± 0.089	–	–	–	–
Epididymides	0.243 ± 0.016	0.246 ± 0.014	0.245 ± 0.021	0.228 ± 0.024	–	–	–	–
Ovaries (10^{-3}%)	–	–	–	–	28.447 ± 3.037	27.333 ± 4.410	28.503 ± 5.596	30.356 ± 3.078

All values represent the mean ± SD.
+, significantly different from the control group at $p \leqq 0.05$ (Mann–Whitney's U-test).
[a] Significantly different from the control group at $p \geqq 0.05$ (Dunnett's test).

Table 10
Histopathological findings of male and female rats following 90-day gavage administration of AHCC-FD.

		Male				Female			
Dose group (mg/kg body weight/day)		0	1000	3000	6000	0	1000	3000	6000
Number of animals examined		10	10	10	10	10	10	10	10
Organ: finding(s)	Grade								
Lung: mineralization, artery	+	0	–	–	0	0	–	–	2
Stomach, limiting ridge: Squamous cell hyperplasia	+	0	0	0	10	0	0	0	3
Hyperkeratosis	+	0	0	0	3	0	0	0	3
Cyst, squamous cell	<+>	1	0	0	1	0	0	0	0
Glandular stomach: ectopic glandular tissue, submucosa	<+>	0	–	–	1	0	–	–	0
Erosion	+	0	–	–	0	0	–	–	1
Ileum: diverticulum	<+>	0	–	–	0	0	–	1(1)	0
Pancreas: atrophy, acinar cell, focal	+	0	–	–	1	1	–	–	0
Liver: Fatty change, centrilobular	+	0	0	0	5	0	0	0	0
Fatty change, periportal	+	0	0	0	0	0	1	0	0
Microgranuloma	+	3	1	5	1	7	4	6	5
Fibrosis, capsule	+	0	0	0	0	0	0	0	1
Heart: myocardial degeneration, focal	+	0	–	–	1	0	–	–	0
Kidney: hyaline droplet, proximal tubular epithelium	+	10	10	10	10	0	0	0	0
Eosinophilic body, proximal tubular epithelium	+	10	10	10	10	0	0	0	0
Regeneration, tubular epithelium	+	4	3	5	4	1	0	0	0
Cast, hyaline	+	1	0	2	1	0	0	1	0
Cellular infiltration, inflammatory cell, renal pelvic mucosa	+	0	0	0	0	0	0	0	1
Hemorrhage, papilla	+	0	0	0	0	0	0	0	1
Mineralization, cortex	+	0	0	0	0	0	0	1	0
Mineralization, cortico-medullary junction	+	0	0	0	0	0	0	2	0
Pituitary: tubular hyperplasia, pars nervosa	+	1	–	–	0	0	–	–	0
Cyst, pars distalis	<+>	0	–	–	1	1	–	–	1
Eyeball: detachment, corneal epithelium	+	0	1(1)	–	0	0	–	–	0
Atrophy, retina	+	0	–	–	1	2	–	–	2
	++	0	–	–	0	0	–	–	1
3rd digit of forelimb: necrosis, subcutaneous tissue	++	–	–	–	0	–	–	–	1(1)
Granulation	++	–	–	–	0	–	–	–	1(1)
Scab	+	–	–	–	0	–	–	–	1(1)

Values represent number of animals with findings. Values in parentheses represent the number of animals examined.
–, blank value.
Grades: +, slight change; ++, moderate change; <+>, presence in "presence or not" basis.

and ALP, were observed among the participants following completion of the 14-day exposure period. Two subjects dropped out because of nausea and intolerance to the test article. Four subjects completing the study reported mild transient adverse events. Terakawa et al. (2008) evaluated the immunological effect of AHCC in a group of 21 healthy subjects randomized into groups receiving 3 g AHCC-FG/day or a placebo for a period of four weeks. Safety-related analyses were included as secondary endpoints in the study, and the authors reported that blood examination results were within normal reference values in both groups.

To further develop the body of evidence supporting the safety of AHCC for potential use as a dietary supplement ingredient, a series of studies evaluating the toxicity of AHCC-FD were conducted. Although the powdered form of AHCC (AHCC-FD) was evaluated in these toxicological studies, the findings are nevertheless relevant to the safety assessment of the granular form that is used clinically. The granular form, AHCC-FG, comprises 60% powdered AHCC and is reformulated with candelilla wax and microcrystalline cellulose to produce an insoluble product. The inclusion of these common food additives as carriers for AHCC powder does not raise toxicological concerns.

AHCC-FD was demonstrated to be non-mutagenic in the standard Ames assay in both the presence and absence of metabolic activation. In the micronucleus assay, administration of AHCC-FD at dose levels up to 2000 mg/kg body weight/day for two consecutive days to mice did not result in an increase of micronuclei incidence in comparison to the vehicle control. AHCC-FD exhibited a very low order of toxicity in the single dose toxicity study. The observation of brown-colored urine in a single AHCC-FD-treated female was likely attributable to the test compound as it is a brown-colored powder that is highly water soluble. No remarkable changes were observed at necropsy with the exception of renal pelvis dilatation in one treated female. The acute oral LD_{50} for AHCC-FD was concluded to be greater than 12,500 mg/kg body weight, the only dose that was evaluated.

In the 90-day repeated dose toxicity study, administration up to 6000 mg/kg body weight/day of AHCC-FD to rats did not produce notable changes in overall condition, body weight, or food consumption when compared to the control group. Ophthalmological and hematological findings were unremarkable. Slight, but statistically significant, increases in serum levels of γ-globulin and creatinine in female and male rats of the high-dose group, respectively, were considered incidental as corresponding histopathological changes attributable to AHCC-FD treatment were not detected in the kidneys. Significant increases in serum total protein levels observed in low-dose female rats and in serum triglyceride concentrations in all AHCC-FD-treated females were neither dose-dependent nor detected in male rats. The statistically significant changes in organ weights, decreased relative brain and adrenal weights for low-dose females and mid-dose males, respectively, were not dose-dependent and thus were considered toxicologically insignificant.

The variation in urinary pH was dose-dependent and likely related to a physiological effect of AHCC-FD (pH 3.7–4.5) administration given its acidic nature. Reduced pH of the urine is a well-described phenomenon following the administration of acidifying substances (Lina and Kuijpers, 2004; Reisinger et al., 2009). The increased urinary protein concentration noted also in the high-dose animals and in the mid-dose females was linked clearly with decreased urinary pH. Given that other measures of kidney function, including kidney weight, kidney histopathology, serum electrolyte, creatinine and BUN values, and other urinalysis

parameters were not adversely affected by treatment, the finding of increased urinary protein in high-dose males and in the top two dose group females was not considered to be toxicologically significant. It is possible that the increased protein load of the diet (AHCC is 11% to 14% protein) could have in some part accounted for this effect. Diets very high in protein content (ca. 50%) are known to increase urinary protein in normal healthy rats (Rumsfeld, 1956). Given the urinalysis findings, the kidneys of all dose groups were subjected to histopathological examination. There was no indication of any adverse effect on kidney pathology (see Table 10). A review of the kidney histopathology slides by Sapporo General Pathology Laboratory, Co. of Japan found no evidence of treatment-related toxicological effects on the kidney at doses of up to 3000 mg/kg body weight/day. One possibly treatment-related finding was the observation of vascular dilatation of the glomerulus in 2/10 males treated at 6000 mg/kg body weight/day.

No compound-related adverse changes in macroscopic and microscopic pathology were observed for the low- and mid-dose groups. The observation of a black patch in the mucosa of the glandular stomach in the high-dose group was unlikely to be related to AHCC-FD administration as it was only observed in one female rat. The finding of slight squamous cell hyperplasia of the limiting ridge of the stomach accompanied by slight hyperkeratosis in the high-dose groups may in part be related to gastric inflammation caused by the low pH of AHCC. A careful examination of the kidney histopathology data in relation to the urinalysis findings (decreased urinary pH and increased protein content) demonstrates no adverse effect of AHCC-FD to a dose of at least 3000 mg/kg body weight/day. Slight fatty changes also were observed in the centrilobular hepatocytes of five high-dose group males.

Based on the aforementioned results of the 90-day toxicity study, the no-observed-adverse-effect level (NOAEL) for AHCC-FD was 3000 mg/kg body weight for both male and female rats under the conditions of this study. A similar NOAEL of 5% in the diet was determined from a 90-day subchronic toxicological evaluation of a mushroom extract derived from *Agaricus blazie* Murrill (Kuroiwa et al., 2005). The extract (ABM-EG3) was produced using hot water and solvent extraction methods and was administered to male and female F344 rats at doses equivalent to 2654 and 2965 mg/kg body weight/day for males and females respectively. Serum biochemistry, hematology and organ weight measurements, and histopathological findings did not reveal any compound-related adverse effects. In contrast, a lower NOAEL of 1000 mg/kg body weight/day was established for a different *A. blazie* Murrill extract (ABM-FD, a water extract) during a 13-week oral gavage toxicity study conducted using SD rats (Sumiya et al., 2008). Dose levels of 500, 1000, or 2000 mg ABM-FD/kg body weight/day were evaluated. Significant changes in several hematology and biochemistry parameters observed in both male and female rats at the high-dose (5000 mg/kg body weight/day) were considered to be suggestive of low level chronic toxicity. Statistically significant and dose responsive increases in plasma creatinine and blood urea nitrogen levels noted in all high-dose animals indicated an effect on the kidney; however, no corresponding changes in histopathology or urinalysis were observed. For females of the high-dose group, significant reductions in total leukocytes, lymphocytes, and large unstained cells were noted at weeks 7 and 13. Absolute and relative spleen weights also were significantly decreased in high-dose females only. The high-dose was established as the lowest-observed-effect level (LOEL) for this study.

The test articles used by Sumiya et al. (2008) and Kuroiwa et al. (2005) were prepared using different extraction processes, and using a different mushroom species from that used to prepare AHCC-FD. Mushrooms contain a multitude of fungal metabolites, the production of which in many cases is species specific. Thus, given that each mushroom extract is distinctive in composition, the ability to compare the findings of studies conducted with test articles prepared using differing extraction methods and from different mushroom species is limited. Nevertheless, the data were presented for completeness to consider the findings of toxicology studies conducted with "similar" test preparations as available in the literature.

In summary, the potential *in vitro* and *in vivo* genotoxic effects and acute and subchronic oral toxicity of AHCC-FD were investigated. AHCC-FD demonstrated a lack of mutagenic activity in the Ames assay and did not induce micronuclei in the bone marrow cells of ICR mice. The acute oral LD_{50} of AHCC-FD was determined to be greater than 12,500 mg/kg body weight. Toxic effects attributable to AHCC-FD exposure were not observed at the low- and mid-dose level groups of the 90-day study whereas compound-related histopathological changes in the limiting ridge of the stomach and in the liver of the high-dose group were noted. Therefore, the mid-dose tested, 3000 mg/kg body weight/day was considered to be the NOAEL for male and female rats. These fundamental toxicology studies provide critical evidence in support of the safety of AHCC consumption.

Conflict of interest statement

The authors of this publication have declared that they have no conflicts of interest.

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