Safety Assessment of Cellulose Nanofibers Obtained from Soda Cooking Bamboo Pulp : Bacterial Reverse Mutation Test, Mouse Lymphoma Thymidine Kinase Assay, Micronucleus Test, and Repeated dose 90-day Oral Toxicity Study in Rats

Tomoko Shimokawa^{*2}, Kengo Magara, Tsutomu Ikeda and Noriko Hayashi

Department of Forest Resource Chemistry, Forestry and Forest Products Research Institute^{*1}

Mutsumi Ogawa, Tetsuya Takao and Eiko Nakayama

Graduate School of Life Sciences, Showa Woman's University

Tomoko Shimokawa

Abstract

Bamboo plants, especially Moso bamboo (*Phyllostachys edulis*), grow rapidly and are suitable resources for reuse loop. With the aim of utilizing Moso bamboo for future application in food materials, cellulose nanofibers (CNFs) were prepared from bamboo pulp via alkaline cooking with wet milling after an enzymatic pretreatment. The certification test revealed that bamboo CNFs were more nano-sized than microfibrillar cellulose which is a recognized food additive. Accordingly, the CNFs cannot be classified into the microfibrillar cellulose category. Therefore, we conducted bacterial reverse mutation test, *in vitro* mouse lymphoma TK (thymidine kinase) assay, and *in vitro* cell micronucleus test on the Chinese hamster lung cell line and confirmed that the bamboo CNFs exhibited no mutagenicity. Furthermore, a repeated dose 90-day oral toxicity study was conducted on rats using 1 wt% bamboo CNFs suspension, which revealed no abnormality on long-term ingestion. No deaths were observed in any of the administration groups throughout the test period and no effects of the test substance were observed on rats under general conditions, urinalysis, and ophthalmologic examination. In addition, hematological examinations, blood biochemical examinations, organ weight measurements, necropsy findings, and histopathological examinations performed after the administration period showed no effects of the test substance on the rats. Since no toxicity due to the test substance was observed in either the male or female administration group, the no observed adverse effect level (NOAEL) under the test conditions was determined to be 200 mg/kg/day or more for both males and females.

^{*1} Matsunosato, Tsukuba-shi, Ibaraki 305-8687, Japan

^{*2} E-mail : tshimo@ffpri.affrc.go.jp

Keywords : cellulose nanofiber, bamboo, mouse lymphoma thymidine kinase assay, micronucleus test, repeated dose 90-day oral toxicity study

1. Introduction

Moso bamboo is a rapidly growing, large-sized bamboo that is distributed mainly in Southeast Asia. The area of the bamboo field in Japan is estimated to be 167,000 ha¹⁾. Bamboo shoots have been used as food materials, and bamboo has been used in the agriculture and fishing industries. Considering its extremely fast growth, moso bamboo is a promising natural resource ; however, its rapid growth often causes erosion into neighboring forests from a lack of proper management. The reason for the increase in abandoned bamboo fields is the decline in demand for bamboo shoots and bamboo materials as well as a shortage of field managers. Therefore, the development of new uses for this fast-growing resource is necessary to increase the economic value of moso bamboo.

Since moso bamboo has a history in the food industry as a bamboo shoot, we focused on the dietary use of cellulose nanofibers (CNFs) produced from bamboo pulp as a new application. Bamboo pulp was prepared by the soda cooking method using moso bamboo as a raw material. Bamboo CNFs were then produced by nano-sizing using wet milling after enzymatic pretreatment. The resulting CNFs showed a cream-like smooth property, and the LD₅₀ was 2,000 mg/kg or more in an acute oral toxicity test in mice²⁾. Supplementing foods with CNF, which is a finely divided cellulose, improves various physical characteristics, such as improvement of dispersion stability and smooth oral texture³⁾. However, confirmation of the safety of the fibers crushed to a nano level is important before using as a food material⁴⁾. In Europe, foods that have not been consumed prior to 1997 are considered new foods, and a safety confirmation is also required for nano-sized foods⁵⁾. Therefore, demonstrating the safety of bamboo CNFs is essential in its development as a future food material. In the present study, we tested the conformity of bamboo CNFs produced from moso bamboo to the standard of fine fibrous cellulose, which is recognized as a food additive. Next, to confirm the safety of bamboo CNFs, a bacterial reverse mutation test, which is a basic toxicity screening test, was performed. An *in vitro* mouse lymphoma thymidine kinase (TK) test using cultured mammalian cells and an *in vitro* micronucleus test using Chinese hamster cells were also performed. In addition, a repeated dose 90-day oral toxicity study in rats was conducted to confirm long-term ingestion and safety.

2. Materials and methods

2.1 Preparation of bamboo CNFs from bamboo soda-pulp

Moso bamboo (*Phyllostachys edulis*) was harvested from a bamboo field in Ushiku City, Ibaraki Prefecture, in November, and then dried at room temperature for 1 month. Bamboo cutting chips (5 cm×3 cm×0.5 cm) were prepared by dividing the part excluding the knots in the direction of the fiber. The bamboo chips (300g as oven dry weight, ODW) were placed into an autoclave (Nitto Autoclave, NAC13, 2 L), and then 900 mL of a cooking solution containing 26% NaOH per chip was added. The mixture was heated to 155 °C over 90 min and maintained at that temperature for 120 min. After the cooking, black liquor was removed by a 300-mesh screen from the cooked chips. The cooked chips were defibrated using a disintegrator (Kumagai Riki No. 2530) for 60 s and then screened using a flat screen (Kumagai Riki No. 2625, 8/1,000 inch wide slit). The pulp was dehydrated to a concentration of approximately 30% by a centrifuge (Kokusan H-130G). Oxygen bleaching and subsequent hypochlorite bleaching of the pulp was carried out as follows. The pulp (120 g as ODW) was mixed with NaOH aqueous solution to prepare a 10% pulp slurry containing 4% NaOH on pulp weight, and placed into a pulp mixer (Quantum, Mk. 5). The temperature of the mixer was increased to 90°C with stirring at 600 rpm for 30 s every 5 min. After the temperature was raised, oxygen gas was introduced at a pressure of 0.6 MPa while stirring at 1,600 rpm for 30 s to mix the oxygen and the pulp slurry. Then, while the oxygen pressure was maintained at 0.6 MPa, the mixture was stirred at 600 rpm for 30 s every 5 min, and the oxygen bleaching was performed for 60 min. After oxygen bleaching, the pulp was washed with water on a 300-mesh screen until it reached neutral pH and then centrifuged to a concentration of approximately 30%. The oxygen-bleached pulp (100g as ODW) was mixed with a NaOH aqueous solution to prepare 10% concentration of pulp slurry of pH 12.0–12.5. The slurry was placed in a plastic bag, and then a sodium hypochlorite solution containing 3% effective chlorine per pulp was added. After the bag was sealed, the slurry was heated for 60 min with occasional stirring in a hot water bath at 60 °C. After the hypochlorite bleaching, the pulp was washed with water on a 300-mesh screen until reaching neutral pH and then dehydrated by centrifugation to obtain the bleached bamboo pulp. The Kla-

son-lignin content of the bleached pulp was 0.1% or less per pulp. The pulp yield after alkaline cooking was 29.3%, and the yield after bleaching was 24.2%.

The 1 wt% pulp slurry was prepared using ion-exchanged water, and cellulase preparation (XL531, Nagase & Co.) was added at a rate of 1 mg (equivalent to protein) per gram (ODW) of bleached pulp. The enzymatic reaction was done at 45°C for 6 h and stopped by thermal treatment at 105°C for 10 min. After enzymatic treatment, the pulp slurry was subjected to a circulating bead mill (LMZ10, Ashizawa Finetech) equipped with zirconia beads (ϕ 1 mm). The bead filling rate was 85%, the peripheral speed of the beads was 12 m/s, and the flow rate was set to 10 L/min. After passing through the entire amount once in a batch process, treatment was carried out by a circulation process. Milling was done to achieve a Dv 90 below 100 μ m as measured by a particle size analyzer (LA-950, Horiba). Dv 90 was defined as the diameter at which 90% of the population counted by volume was below this value²⁾. The resulting bamboo CNFs were packed in a bag in 1 kg aliquots and sterilized at 105 °C. The morphology of the prepared CNF was assessed with an optical microscope and a transmission electron microscope (TEM, JEM-2000EX, JEOL). The product exhibited a width of 3–100 nm, a length of 1 μ m or more, and a branched shape (Fig. 1).

2.2 Property test

To investigate whether bamboo CNFs conform to the standard⁶⁾ of the existing food additive, microfibrinous cellulose, as specified in the 8th Edition Food Additives Official Standard, a property test of (1)–(4) was performed. The CNF slurry was condensed to 10 wt% by heating at 70°C and sterilized by autoclave as a test specimen, and the appearance was observed visually. For the confirma-

Fig. 1 Transmission electric microscopy (TEM) image of bamboo cellulose nanofibers (CNFs) obtained by enzymatic hydrolysis followed by mechanical treatment

tion test (1), the test specimen was dried, finely cut or loosened on a thin film, and measured by the potassium bromide tablet method using infrared absorption spectrum analysis. For the test (2), 5.0 g (ODW) of the specimen was diluted to 100 g with water and homogenized using a homogenizer equipped with a blade diameter of approximately 35 mm in a cup (150 mL). The appearance of the mixture was observed after forcible mixing at 10,000–12,000 rpm for 3 min and reconfirmed after 3 h. In the test (3), 1.0 g (ODW) of the specimen was diluted to 100 g with water, and the solution was stirred at 10,000–12,000 rpm for 3 min using the same homogenizer described above (2). The resulting cloudy slurry was placed on a 25 μ m standard sieve (20 cm in diameter) with a receiver and gently vibrated laterally for 10 s. The filtered transparent or cloudy solution was evaporated to dryness and the amount of residue was measured. For purity assessment, the following specified tests (1) to (4) were carried out. (1) The solution pH, in which 2.0 g of the test specimen was suspended in 100 mL of water, was measured. The contents of (2) lead and (3) arsenic were measured by the specified methods, and the amount of lead in 5.0 g (ODW) of the specimen and the amount of arsenic in 1.0 g (ODW) were calculated. (4) For the measurement of water-soluble substances, 200 mL of water was added to 4.0 g (ODW) of the test specimen. The slurry was stirred with a high-speed disperser ((DH-2.5, Primix) equipped with clover blades (four blades with a length of about 13 mm and a maximum width of about 16 mm) at 5,000 rpm for 5 min. The dispersion liquid was suction-filtered through filter paper for quantitative analysis (Type 5 C), and 50 mL of the filtrate was evaporated to dryness in a water bath. The resulting residue was dried at 120°C for 1 h, allowed to cool in a desiccator, and then weighed. For the measurement of dry weight loss, 5 g of the test specimen was incubated at 120°C for 5 h. The ash content was measured using 2.0 g (ODW) of the test specimen. The microbial limit was confirmed by a pour plate method using CompactDry “Nissui” for total viable cell count and for coliform and *Escherichia coli* count using bamboo CNFs sterilized by autoclave. The sample confirmation tests (1)–(3), the purity tests (1)–(3), drying weight loss, and ash content measurement were carried out by the Japan Food Hygiene Association.

2.3 Bacterial reverse mutation test

Salmonella Typhimurium (histidine auxotroph : TA98, TA100, TA1535, TA1537) and *Escherichia coli* (tryptophan auxotroph : WP2uvrA) were used for the reverse mutation test. Based on the “Guidelines for the Designation of Food Additives and Revision of Standards for Use of Food Additives (Notification No. 29, March 22, 1996)”

by the Ministry of Health, two cases were done in the presence (+S9 mix) and absence (–S9 mix) of activation using rat–liver homogenates (S9) to examine the mutagenesis of bamboo CNFs. The bamboo CNFs were condensed to 5 wt% by heating at 70 °C and sterilized with an autoclave. The specimen was diluted with injection solvent to a range of concentrations (8.19–2,000 µg/mL). The following chemicals were used as positive controls : 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2), sodium azide (NaN₃), 9-aminoacridine hydrochloride (9-AA), and 2-aminoanthracene (2-AA). The test employed a pre-incubation method in which 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) (absence of metabolic activation) or 0.5 mL of S9 mix (presence of metabolic activation), and 0.1 mL of the test substance (solvent, test specimen or positive control) were added to a test tube. The bacterial inoculate (0.1 mL) was then added and incubated for 20 min at 37°C. After incubation, 2 mL of top agar (0.6% soft agar medium containing 0.5% sodium chloride containing L-histidine for *S. Typhimurium* and L-tryptophan for *E. coli*) was added and mixed. The plates were incubated for 48 h at 37 °C. Following incubation, the number of colonies was determined. The test was conducted at the Public Interest Incorporated Foundation BioSafety Research Center (BSRC).

2.4 *In vitro* mouse lymphoma TK assay

Gene mutagenesis of bamboo CNFs was examined by the mouse lymphoma TK assay using mouse lymphoma cells (L5178Y tk^{+/–}–3.7.2c). The mouse lymphoma TK assay is based on the fact that cells carrying the thymidine kinase gene are sensitive to trifluorothymidine (TFT), which inhibits metabolism and cell division, whereas mutant cells are resistant to TFT.

As the test specimen, the CNF slurry was concentrated to 5 wt% at 70 °C and sterilized by autoclaving. The assay was carried out in reference to OECD guidelines (TG 490) for *in vitro* mammalian cell gene mutation test using the TK gene (28 July, 2015). Cell suspensions (10⁶ cells/mL) were prepared in RPMI 1640 medium (Wako) containing 10% deactivated horse serum, 500 units/mL penicillin, 5,000 µg/mL streptomycin, and 100 mM sodium pyruvate at 37 °C. The cells were cultured under conditions of high humidity with a CO₂ concentration of 5%. The medium containing 10% horse serum described above was designated R-10 medium, and the medium containing no horse serum and containing 20% was designated R-0 and R-20 media, respectively. Injection solvent was used as a negative control and to dilute the test specimens to a dose of 0.0781–20 mg/mL. The positive control substances were methyl methanesulfonate (MMA) and cyclophosphamide (CP) dissolved in physiological saline. The cell suspension was incubated for ei-

ther 3 h for short-term treatment or 24 h for continuous treatment and were performed in the presence (+S9 mix) or absence (–S9 mix) of metabolic activation. After treatment, the cells were collected by centrifugation, washed, and resuspended in R-10 medium to a concentration of 2×10⁵ cells/mL. The cell suspension was cultured for 48 h to allow for expression of the mutant phenotype and then diluted to 1×10⁴ cells/mL using R-10 medium for the dose-finding study (15.6–20 µg/mL) and R-20 for the main study. TFT was added to the cell suspension (1×10⁴ cells/mL) at 3 µg/mL for the selection of mutant colonies. The cell suspension (0.2 mL) was transferred to 96-well plates and incubated for 12 h at 37 °C. After incubation, the size of the colonies was evaluated by assuming that the small colonies were smaller than 1/4 of the well diameter and the large colonies were 1/4 or more of the well diameter using a stereomicroscope for each plate. The number of wells containing only large colonies, small colonies, both types, and no colonies was counted to determine mutation frequency (MF). In addition, an aliquot of each cell suspension was diluted to 8 cells/mL and transferred to a 96-well plate (0.2 mL/well) and incubated for 12 h at 37°C to measure the cloning efficiency (CE). Relative cloning efficiency (RCE) was calculated by dividing the CE of the test specimen for each dose by that of the negative control.

Based on the background data, the criteria for determining the test result as positive were as follows. The total mutation frequency (T-MF) obtained by treatment with the test substrate exceeded the value obtained by adding 126×10^{–6} to that of the negative control and when a dose dependency was observed in the increased frequency of occurrence. In addition, we determined that the test would be successful if the following parameters were satisfied. 1) T-MF of the negative control was 50×10^{–6} to 170×10^{–6}, and CE was 65–120% ; 2) the total suspension growth (Total SG) of the negative control was 8–32 for short-term treatment and 32–180 for continuous treatment ; 3) T-MF of the positive control is increased by 300×10^{–6} or more compared with that of the negative control, the rate of gene mutation frequency (% SC) resulting in small colonies is 40% or more, or S-MF of the positive control increases by 150×10^{–6} or more compared with that of the negative control ; and 4) the relative total growth (RTG) of the positive control is 10% or more, and T-MF is within the fluctuation range of the background data. This test was carried out at the CMIC Pharma Science Co., Ltd.

2.5 *In vitro* Chinese hamster cell micronucleus test

A genetic toxicity test was conducted to evaluate the chromosomal structure abnormality and aneuploidy induction of the test specimen by detecting micronuclei in

the cytoplasm of interphase cells using Chinese hamster lung-derived fibroblasts (CHL/IU). The test was carried out in reference to the OECD guidelines (TG 487) for the *in vitro* mammalian cell micronucleus test (September 26, 2014). Using sterilized 1 wt% bamboo CNF as the test specimen, BS-MEM culture medium, which contained 10% inactivated horse serum in the Minimum Essential Medium (MEM, Gibco), was added to the cell culture and used for the preparation of the test substance (625–5,000 $\mu\text{g/mL}$). BS-MEM was used as a negative control and CP dissolved in physiological saline was used as the positive control. In the case of short-term treatment with metabolic activation using S9 (+S9 mix), the cells were washed with physiological saline after treatment for 6 h and resuspended in new medium for an additional 24 h of incubation. No metabolic activation was performed during the 24 h of continuous treatment. After incubation, the state of the cells was confirmed with an inverted phase-contrast microscope, the cells were treated with a 0.25% trypsin solution, and an aliquot was used to measure cell concentration. Additionally, 1,000 mononuclear cells were observed at each dose for the occurrence of micronuclei after staining with Giemsa solution. The test was carried out at the BoZo Research Center Co., Ltd.

2.6 Repeated dose 90-day oral toxicity study in rats

Bamboo CNF was orally administered to male and female SD rats at doses of 0, 50, 100, and 200 mg/kg for 90 days to evaluate toxicity according to the “Guidelines for the Designation of Food Additives and Revision of Standards for Use of Food Additives (Notification No. 29, March 22, 1996)” by the Ministry of Health. Sterilized 1 wt% bamboo CNF was used as the test specimen to avoid the possibility of agglomeration resulting from concentration of the CNF slurry.

Male (100–160 g) and female (80–130 g) 5-week-old Crl:CD (SD) rats (specific pathogen-free) were obtained from Charles River Laboratories Japan and housed for 6 days to acclimate. The breeding environment was set to a temperature of 20–26°C, a humidity of 35–70% RH, with a ventilation rate of 12 times or more per hour. A light timer was set to 12 h each for light (7 : 00–19 : 00) and dark. CRF-1 (Oriental Yeast Co., Ltd.) was freely ingested except during fasting, and tap water was provided from an automatic absorption nozzle. All rats were weighed and assessed for physical abnormalities, general condition, body weight transition, and ophthalmologic health during the acclimatization period. They were grouped on the administration start date (day 1), and each group contained 10 males and females. The maximum dose was set to 200 mg/kg/day, which corresponded to “in the case of aqueous solution where 2 mL/100 g

body weight may be used” described in the OECD guideline (TG 408). For medium and low doses, 100 and 50 mg/kg/day were used, respectively. The dose volume for all groups was 20 mL/kg, the injection solvent was used for dilution and control, and the test substance was orally administered once daily using a gastric tube.

During the acclimatization period, ophthalmologic examinations were performed to exclude defective animals from the test system. The ophthalmologic examinations were also performed on five animals from each group in ascending order of animal number during the final week of administration (day 86). For the ophthalmologic examination, the appearance and light reflex were confirmed, and the anterior segment of the eye, intermediate translucent body, and fundus were examined after treatment with a mydriasis agent.

All animals were observed daily for general condition and body weight, and food intakes were measured every 3 or 4 days from day 1. Additionally, at the end of the observation period (day 91), hematological and blood tests were performed on all survivors. Urinalysis was performed on days 87–88 (the last week of administration) for five animals with younger animal numbers in each group. For all animals in each group, rats fasted after 17 : 00 on the day before blood collection and were laparotomized under isoflurane anesthesia, and blood was obtained from the abdominal aorta.

For the hematological examination, a portion of blood was dispensed into a blood collection tube containing an anticoagulant (EDTA-2K), and hematocrit (HCT), hemoglobin amount (HGB), red blood cell count (RBC), mean corpuscular volume (HCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte rate, reticulocyte count, platelet count (PLT), white blood cell count (WBC), white blood cell percentage, neutrophil (NEUT), lymphocyte (LYMPH), monocyte (MONO), eosinophils (EOSN), and basophils (BASO) were measured using a comprehensive hematology tester (ADVIA 120, Bayer). The prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen amount were measured by withdrawing blood in a blood collection tube containing an anticoagulant (3.2% sodium citrate aqueous solution) and centrifuging at $1,700 \times g$ for 13 min. The resulting plasma was analyzed by a fully automatic blood coagulation/fibrinolysis measuring device (STA Compact, Roche).

For biochemical tests, the serum was obtained from each blood sample, which was collected into a blood collection tube (Venoject II, Telmo) containing a coagulation-promoting film and a dispersant, and centrifuged at $1,700 \times g$ for 13 min. Total protein, glucose, triglycerides,

total cholesterol, blood urea nitrogen (BUN), creatinine, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyltranspeptidase (Gamma-GTP), calcium, inorganic phosphorus, albumin fraction ratio, and globulin fraction ratio were all measured using a multi-item biochemical automatic analyzer (Hitachi 7170, Hitachi, Ltd.). Sodium, potassium, and chloride were measured using an automatic electrolyte analyzer (EA06R, A&T). In addition, the albumin fraction concentration (fraction ratio $\times$ total protein concentration/100) and A/G (albumin fraction ratio/total globulin fraction ratio) were obtained using the above calculation.

Fresh urine samples were used to measure pH, occult blood, ketones, glucose, protein, bilirubin, and urobilinogen using Ames test paper for urine (N-Multistix SG-L, Siemens Healthcare Diagnostics) with an automatic urine analyzer (CLINTEK Advantus, Siemens Healthcare Diagnostics).

For pathological examinations, organ weight measurement, macroscopic examination (necropsy) of various organs, and histopathological examinations were performed. Animals were euthanized as described above after blood samples were collected. The necropsies included examination of the external surface, all orifices, and the systematic organs and tissues including the abdominal, thoracic, pelvic, and cranial cavities. Simultaneously, the following tissues and organs were weighed: brain, heart, liver, spleen, kidneys, adrenal gland, testes, ovaries, lungs, uterus, pituitary gland, thymus, prostate, seminal vesicles, salivary gland, and thyroid gland. For the prostate, seminal vesicles, pituitary gland, and thyroid gland, the organ weight was measured after fixing in a 10 vol% of neutral buffered formalin solution. The ratio of organ to body weight was calculated from the body weight on the day of necropsy.

In addition to the weighed organs, the following organs and tissues with macroscopic abnormalities were fixed with 10 vol% of neutral buffered formalin solution: skin, mammary gland, pancreas, lymph nodes, sternum, urinary bladder, femur (left), bone marrow, trachea, epididymides, vagina, parathyroid, eyeballs, tongue, harderian gland, esophagus, stomach, duodenum, spinal cord, jejunum, skeletal muscle, ileum, sciatic nerve, cecum, aorta, colon, oviduct, rectum, Zymbal gland, and nasal cavity. The testis was pre-fixed with formalin acetate, whereas the eyeballs and harderian gland were pre-fixed with Davidson's solution. These samples were embedded in paraffin, sliced, and stained with hematoxylin and eosin for histopathological examination.

Bartlett's test of homogeneity of variances was first performed for body weight, food intake, hematological

examination, blood biochemical examination, organ weight, and the ratio of organ weight to body weight. In the event of homoscedasticity, Dunnett's multiple comparison tests was used to show significant differences between the control group and each administration group. In the case of unequal dispersion, a significant difference was assessed using Steel's test. The significance level was 5% for Bartlett's test and 5% and 1% for the other tests.

The test was conducted at BSRC and approved by the Animal Research Committee of Forest and Forest Products Research Institute (FFPRI) and the BSRC in compliance with the following ethical guidelines: "Guideline for Conducting Animal Experiment etc. in Forestry and Forest Products Research Institute" and "Guidelines for Animal Experiments" (June 2, 2014) at the BSRC.

3. Results

3.1 Property test

The appearance of microfibrinous cellulose, which is a food additive, was defined as white moist and cotton-like, whereas that of the bamboo CNF (10 wt%) was like moist clay and pale yellow. In contrast, the bamboo CNF showed conformity to the standards of the confirmation tests from (1) to (3), which indicates that (1) infrared absorption spectrum analysis showed a similar intensity absorption at the same wave number compared with the reference spectrum; (2) when the mixture was forcibly stirred by a homogenizer in the dispersion state confirmation test, the mixture became a white opaque dispersion, and this state was maintained without separation even after 3 h; (3) in a test to confirm the amount of residual substance after sieving, the results conformed to the specified value of 0.3 g or less. The results of the purity test were as follows. (1) The specified pH range was 5.0–8.0, and the bamboo CNF was within this range. The contents of (2) lead and (3) arsenic were defined as 2.0 $\mu\text{g/g}$ or less for Pb and 2.0 $\mu\text{g/g}$ or less for As_2O_3 ; thus, the bamboo CNF satisfied these values. The amount of water-soluble matter (4), obtained through filter paper for quantitative analysis (Type 5 C), was determined to be 0.50% or less, whereas that of the bamboo CNF specimen was 2.3%, which exceeded the specified value. It should be noted that CNFs with a width of 3–4 nm and a length of 0.3–1 μm or more were observed when the filtrate was examined with a TEM (Fig. 2). Both the dry weight loss and the ash content were within the stated ranges of 60.0–92.0% and 0.50% or less, respectively. The microbial limit was less than the specified number of bacteria per gram (5,000 or less) in the sterilized sample, and no *E. coli* was detected.

Fig. 2 TEM image of bamboo CNFs in the filtrate after filtering with filter paper No. 5C

3.2 Bacterial reverse mutation test

No clear increase in the number of reverted mutant colonies was observed in the bamboo CNF-treated group compared with the negative control group in the reverse mutation test using *S. Typhimurium* and *E. coli*, regardless of the presence or absence of metabolic activation (Table 1). Precipitation of the test substance was confirmed in both treatments at a concentration of 51.2 $\mu\text{g/mL}$ or higher at the time of colony measurement, but no growth inhibitory effect on the test strain was observed at any dose. In contrast, since the positive control substance significantly induced a return mutation, the gene mutagenesis of the bamboo CNF under the test

conditions was judged to be negative.

3.3 *In vitro* mouse lymphoma TK assay

The relative total growth rate (RTG) was less than 20% at dose levels of 1,000 $\mu\text{g/mL}$ or higher for short-term treatment and 500 $\mu\text{g/mL}$ or higher for continuous treatment for 24 h with or without metabolic activation. Furthermore, since precipitation of the test substance was observed at 62.5 $\mu\text{g/mL}$ or higher, the test was conducted with the maximum dose of 62.5 $\mu\text{g/mL}$ (Table 2). Neither short-term nor continuous treatment showed a decrease in colony formation rate and RTG at either dose. In addition, there was no significant increase in T-MF and no increase in the dose-dependent frequency. In contrast, the T-MF for the positive control increased under all treatment conditions. Both the negative and positive controls satisfied the above-mentioned conditions for establishing the test, indicating that the test was carried out appropriately. Based on these results, bamboo CNF under the test conditions was considered negative.

3.4 *In vitro* Chinese hamster cell micronucleus test

Initially, we planned a study using free-floating human lymphoblastic cells (TK6). However, since the test solution was gel-like, the cells did not settle during centrifugation, making it difficult to collect the cells. Therefore, adhesive CHL/IU cells were used. To obtain the maximum concentration of the test substance recommended by the referenced guidelines, 5,000 $\mu\text{g/mL}$, a culture medium containing bovine serum was used for dose prepa-

Table 1 Results of the primary tests in the bacterial reverse mutation assay of bamboo CNFs

Substance	Dose	Average number of revertants per plate									
		TA98		TA100		TA1535		TA1537		WP2 $uvrA$	
		+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Negative control	0.1 ml/plate	35	23	125	113	12	16	16	10	28	27
Bamboo CNFs	8.19 $\mu\text{g/plate}$	37	26	157	131	14	19	14	9	29	24
	20.5 $\mu\text{g/plate}$	35	23	128	127	11	15	10	6	19	30
	51.2 $\mu\text{g/plate}^a$	34	27	129	125	15	15	16	8	26	29
	128 $\mu\text{g/plate}^a$	40	28	139	124	11	16	11	5	24	37
	320 $\mu\text{g/plate}^a$	34	25	136	129	12	10	17	7	37	29
	800 $\mu\text{g/plate}^a$	34	22	136	118	16	15	10	11	23	19
	2000 $\mu\text{g/plate}^a$	42	15	118	121	12	15	12	4	26	24
	5000 $\mu\text{g/plate}^a$	32	39	144	116	12	13	17	7	32	24
	AF-2	0.01 $\mu\text{g/plate}$			513						101
2-AA	0.1 $\mu\text{g/plate}$		664								
	0.5 $\mu\text{g/plate}$	406									
	1 $\mu\text{g/plate}$			1045							
	2 $\mu\text{g/plate}$					332		196			
9-AA	10 $\mu\text{g/plate}$									835	
	80 $\mu\text{g/plate}$								220		
NaN ₃	0.5 $\mu\text{g/plate}$					574					

The negative control was sterile water for injection.

CNF, Cellulose nanofiber; AF-2, 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide; 2-AA, 2-Aminoanthracene; 9-AA, 9-Aminoacridine.

^aTest substance precipitation.

Table 2 Results of the mouse lymphoma cell thymidine kinase gene mutation assay in L5178Y tk^{+/−} cells exposed by bamboo CNFs

Condition	Substance	Dose (µg/mL)	S9	CE ^b (%)	RCE ^c (%)	Total SG ^d	RSG (%) ^e	RTG (%) ^f	MF _g (×10 ⁶)			%SC ^h
									Total	Large	Small	
Short term treatment (3 h)	Negative control Bamboo CNFs		−	118.1	100	13.7	100	100	149.5	71.3	72.7	48.6
		7.81	−	107.2	90.8	17.0	123.6	112.2	148.6	64.2	81.0	54.4
		15.6	−	123.0	104.1	15.6	113.8	118.5	124.6	47.1	75.1	60.3
		31.3	−	118.5	100.3	17.7	128.8	129.2	119.1	50.8	66.6	55.9
		62.5 ^a	−	109.2	92.5	17.9	130.4	120.6	134.2	46.7	83.0	61.8
	MMS Negative control Bamboo CNFs	20	−	57.3	48.5	10.1	73.7	35.7	1966.9	419.1	1176.6	59.8
			+	98.1	100	15.2	100	100	127.4	84.6	39.3	30.8
		7.81	+	108.3	110.4	14.9	97.7	107.9	126.5	94.5	29.1	23.0
		15.6	+	94.4	96.2	14.4	94.6	91.0	98.2	86.0	14.4	14.7
		31.3	+	81.7	83.3	15.2	99.9	83.2	130.8	109.9	19.1	14.6
CP	62.5 ^a	+	106.6	108.7	13.1	86.2	93.7	108.5	94.7	12.5	11.5	
	3.0	+	48.3	49.2	6.3	41.2	20.3	995.5	454.5	441.0	44.3	
Continuous treatment (24 h)	Negative control Bamboo CNFs		−	101.3	100	68.1	100	100	148.5	82.0	64.3	43.3
		7.81	−	78.2	77.2	81.7	120.0	92.6	158.5	78.2	75.1	47.4
		15.6	−	113.8	112.3	64.5	94.7	106.3	110.8	52.9	54.4	49.1
		31.3	−	109.7	108.3	67.4	98.9	107.1	120.6	58.9	60.0	49.8
		62.5 ^a	−	94.4	93.2	74.2	109.0	101.6	94.8	36.7	56.2	59.3
	MMS	10	−	32.7	32.3	27.0	39.6	12.8	3652.4	1090.3	1694.6	46.4

The negative control was sterile water for injection.
MMS, Methyl methane sulfonate; CP, Cyclophosphamide.
CE, Cloning efficiency; RCE, Relative cloning efficiency; SG, suspension growth; RSG, Relative SG; RTG, Relative total growth; MF, Mutation frequency; %SC, % small colonies.
^aTest substance precipitation.
^bCE=[−ln(number of empty wells/total wells)]/(mean number of seeded cells per well)
^cRCE=(CE of test substance dose/CE of negative control)×100.
^dTotal SG=(growth rate between day 0 and day 1)×(growth rate between day 1 and day 2).
^eRSG(%)=(total SG of test substance dose/total SG of negative control)×100.
^fRTG(%)=RSG×RCE
^gMF=[−ln(number of empty wells/total wells)]/(mean number of cells per well)×(total wells)/[CE×(number of wells when calculating CE)]
^h%SC=(MF of small colonies/Total MF)×100.

Table 3 Results of the *in vitro* micronucleus test in cultured Chinese hamster lung fibroblast cells treated with bamboo CNFs

Substance	Dose ($\mu\text{g/ml}$)	S9 mix	Time ^a (h)	Number of cells (%)			RPD (%) ^b
				Cells with micronucleus	Cells with polynuclear	Cells of metaphase	
Negative control	0	+	6-24	15 (1.5)	11 (1.1)	31 (3.0)	100
Bamboo CNFs	625	+	6-24	13 (1.3)	14 (1.4)	21 (2.0)	89
	1250	+	6-24	15 (1.5)	14 (1.3)	29 (2.8)	94
	2500	+	6-24	19 (1.9)	8 (0.8)	24 (2.3)	111
	5000	+	6-24	20 (2.0)	12 (1.2)	29 (2.8)	-10
	CP	+	6-24	280 (28.0)	134 (11.1)	68 (5.7)	18
Negative control	0	–	24-0	16 (1.6)	12 (1.2)	22 (2.1)	100
Bamboo CNFs	625	–	24-0	17 (1.7)	14 (1.3)	25 (2.4)	86
	1250	–	24-0	18 (1.8)	14 (1.3)	25 (2.4)	103
	2500	–	24-0	11 (1.1)	8 (0.8)	27 (2.6)	91
	5000	–	24-0	19 (1.9)	17 (1.6)	17 (1.6)	-36

^aTime shows treatment time-recovery time.

The number of observed cells at each test article was 1000.

The negative control was medium.

CP, cyclophosphamide; RPD, Relative population doubling.

^bRPD(%)=(population doubling number (PD) of test article $\times$ 100)/(PD of negative control)

PD=log[(final cell number after treatment)/(cell number before treatment)]/log2

ration, and this medium was also used as a negative control. The precipitation of the test substance at all doses for all treatment methods did not interfere with the performance of the micronucleus analysis (Table 3). At the highest dose (5,000 $\mu\text{g/mL}$), the relative cell population doubling (RPD) was -10% under short-term treatment with metabolic activation and -36% under continuous treatment, indicating a suppression of cell proliferation. Although the exact cause of this event has not been clarified, no increase in the frequency of occurrence of cells with micronuclei was observed; thus, we concluded that there was no problem with the appropriateness of the study. For the bamboo CNF, no significant increase was observed in the number of cells with micronuclei when compared with the negative control group using Fisher's direct establishment calculation method in the metabolic activation of the short-term and continuous treatment methods. In contrast, the incidence of cells with micronuclei in the positive control group increased significantly. Therefore, the micronucleus inducibility of bamboo CNF was negative under the test conditions and considered to have no ability to induce chromosomal aberrations.

3.5 Repeated dose 90-day oral toxicity study in rats

3.5.1 Animals excluded from evaluation

One animal in the bamboo CNF 200 mg/kg/day group had an intrathoracic tumor mass adhering to an organ in the abdominal cavity, which was observed at necropsy, and a histopathological examination revealed a foreign-body granuloma with foreign substances (test substances) in the lungs and pleura. Since this foreign-body

granuloma was considered to be a foreign-body reaction to the test substance injected into the thoracic cavity because of an administration error, the data obtained from this animal was excluded from evaluation. Although this one case was defective, toxicity evaluation was possible using the other nine cases in the same group; therefore, the event was judged not to affect the overall reliability of the study.

3.5.2 General condition, body weight, and food intake

No abnormalities in general condition were observed in any of the test groups. A broken maxillary incisor was observed in one animal of a control group from day 32 to day 36.

There was no statistically significant difference in body weight transition and weight gain during the administration period in male rats (Fig. 3), although the 100 and 200 mg/kg/day groups tended to have values slightly higher than those of the control group throughout the administration period. In the case of female rats, body weight showed a statistically significant increase in the 50 mg/kg/day group from day 81 to day 87, and the amount of weight gain was also increased during the administration period compared with that of the control groups. Since no statistically significant difference was observed in the female 100 and 200 mg/kg/day groups compared with the control group, the higher values at the 50 mg/kg/day group was considered to be an accidental change. However, it was noteworthy that all female test substance-administered groups tended to show

Fig. 3 Body weight changes of rats treated orally with bamboo CNFs in 90-day repeat dose toxicity test. The bars represent $\pm$ SD. Significantly different from 0 mg/kg group, * $p \leq 0.05$.

slightly higher values throughout the administration period.

The mean daily food intake of the male rats was significantly increased between days 28–32, 35–39, 42–46, 49–53, and 53–56 in the 200 mg/kg/day group and days 53–56, 60–63, 67–70, and 81–84 in the 100 mg/kg/day group compared with that of the control groups. The values tended to be slightly higher on other measurement days. In female rats, the food intake of all test substance-administered groups tended to be slightly higher throughout the administration period. Significant increases were observed on days 28–32 and 46–49 in the 100 mg/kg/day group and on days 14–18, 18–21, 28–32, 32–35, 46–49, and 53–56 in the 50 mg/kg/day group (Fig. 4).

3.5.3 Urinalysis and ophthalmic examination

There were no statistically significant differences or significant changes between the test substance-administered group and the control group on urinalysis parameters for both male and female rats (Table 4).

Ophthalmologic observations in the final week of administration revealed corneal and crystalline lens opacity in each of the treatment groups, including control groups, and atrophy of the retinochoroid in one rat of the female 50 mg/kg/day group was observed. Because the abnormalities described above were sporadic or less than that of the control group, and since they were spontaneous changes often observed in rats of the same strain, they were not considered as a notable abnormal case.

3.5.4 Blood test

The results of the hematological tests are shown in Table 5. There were few statistical differences observed in male rats including prolongation of APTT in the 100

Fig. 4 Food consumption of rats treated orally with bamboo CNFs in 90-day repeated dose toxicity test. The bars represent $\pm$ SD. Significantly different from 0 mg/kg group, * $p \leq 0.05$, ** $p \leq 0.01$ (Dunnett).

and 200 mg/kg/day groups, low red blood cell counts in the 100 mg/kg/day group, and high reticulocyte rate. These changes were mild and dose independent. No significant differences were observed between the groups in any of the hematology parameters in the female rats.

With respect to the blood biochemistry examination (Table 6), sodium concentration was significantly higher in the male 200 mg/kg/day group; however, no significant difference was observed between the groups in any of the parameters in female rats. Therefore, no anomaly was evident in the parameters of the blood test.

3.5.5 Pathological examination

Table 7 shows the results of organ weight measurement. The relative weight of the thyroid gland was significantly lower in the male 100 mg/kg/day group, but the decrease was dose independent and no change was observed in absolute weight. The absolute lung weight was significantly higher in the 200 mg/kg/day female rat group. In addition, the absolute weights of the heart and kidneys in the 100 mg/kg/day group and the liver in the 50 mg/kg/day group were significantly higher, but the changes did not correspond to the dose.

Sporadic findings in a small number of cases at necropsy were as follows: swollenness/nodules/white spots of the spleen, brown spots on the lungs, red spots in the nasal cavity, small intestinal nodule, recessed lesions/white spots of the kidney, small testes and epididymis, dilation of the uterine cavity, vaginal nodules, pituitary cyst, small thyroid, and brown spots on the adrenal glands (Table 8). However, all of them were localized and were spontaneous lesions often found in normal rats, and an effect of the test substance was not observed.

Table 4 Urinalysis data of rats treated orally with bamboo CNFs in 90-day repeated dose toxicity test

Sex		Male				Female			
		0	50	100	200	0	50	100	200
Dose level (mg/kg)		0	50	100	200	0	50	100	200
Number of animals		5	5	5	5	5	5	5	4
pH	6.5	0	0	0	0	0	0	1	
	7.0	1	0	0	0	1	0	2	2
	7.5	0	0	1	0	1	2	0	1
	8.0	1	3	2	0	1	1	1	1
	8.5	3	2	2	5	2	2	1	0
Protein (mg/dL)	–	1	2	2	2	5	4	4	3
	+/-	3	2	3	2	0	1	1	1
	30	1	1	0	1	0	0	0	0
	100	0	0	0	0	0	0	0	0
Glucose (g/dL)	–	5	5	5	5	5	5	5	4
	0.1	0	0	0	0	0	0	0	0
Ketones (mg/dL)	–	5	4	5	4	5	5	5	4
	5	0	1	0	1	0	0	0	0
	15	0	0	0	0	0	0	0	0
Bilirubin	–	5	5	5	5	5	5	5	4
	1+	0	0	0	0	0	0	0	0
	2+	0	0	0	0	0	0	0	0
	3+	0	0	0	0	0	0	0	0
Occult blood	–		1	0	1	4	4	3	2
	+/-	5	3	5	4	1	1	2	2
	1+	0	1	0	0	0	0	0	0
	2+	0	0	0	0	0	0	0	0
	3+	0	0	0	0	0	0	0	0
Urobilinogen (EU/dL)	0.1	5	5	5	5	5	5	5	4
	1.0	0	0	0	0	0	0	0	0

Grade: –, negative; +/-, trace; 1+, slight; 2+, moderate; 3+, severe.

EU, Ehrlich Unit.

Table 9 shows the findings of the histopathological examination of planned necropsy. The changes observed only in the test substance-administered group were as follows : necrosis/envelope inflammation of the spleen, lung bleeding, jejunal double cyst, inflammatory cell infiltrate in the mandibular/sublingual glands/kidney/bladder, kidney cyst, seminiferous tubule atrophy of the testis, oligospermia of the epididymis, vaginal cyst, pituitary cyst, remnants of the craninopharyngeal canal, and an irregular retinal arrangement of the eyeball. These were accidental lesions caused by tissue malformations, inflammation, etc., and represent spontaneous lesions that occasionally occur in normal rats. Since other changes occurred in the control group at almost the same frequency, such changes were not considered to result

from the test substrate. In conclusion, there were no notable abnormalities in the histopathological observation.

4. Discussion and conclusion

Bamboo CNFs prepared by an integrated manufacturing process following concentration to 10 wt% was found to be different in appearance and content of water-soluble substances from the fine fibrous cellulose, consistent with the 8th Edition Food Additives Official Regulations. The exterior appearance of the bamboo CNFs was pale yellow, moist clay-like, and less white, and they have a smoother appearance than microfibrillar cellulose (white moist cotton-like). Regarding the color tone, since xylan-related components were found to be mixed in the bamboo pulp²⁾, this effect may have decreased the white-

Table 5 Hematology data of rats treated with bamboo CNFs in 90-day repeated dose toxicity test

Sex	Male						Female					
	Dose level (mg/kg/day)			Number of animals			Dose level (mg/kg/day)			Number of animals		
	0	50	100	200	0	50	100	200	0	50	100	200
	10	10	10	10	10	10	10	10	10	10	10	9
RBC (×10 ⁶ /μL)	8.71 ± 0.40	8.73 ± 0.24	8.29 ± 0.20**	8.50 ± 0.28	8.32 ± 0.28	8.22 ± 0.26	8.26 ± 0.35	8.04 ± 0.19				
HGB (g/dL)	15.0 ± 0.3	15.2 ± 0.4	14.7 ± 0.6	15.0 ± 0.3	15.2 ± 0.5	14.9 ± 0.5	15.1 ± 0.4	15.1 ± 0.4				
HCT (%)	43.9 ± 0.9	44.7 ± 1.0	43.1 ± 1.4	43.9 ± 1.0	44.1 ± 1.4	43.2 ± 1.5	43.9 ± 1.5	43.2 ± 1.2				
MCV (fL)	50.5 ± 1.8	51.2 ± 1.8	52.0 ± 1.8	51.6 ± 1.2	53.0 ± 1.2	52.6 ± 1.2	53.2 ± 1.5	53.8 ± 1.5				
MCH (pg)	17.2 ± 0.7	17.4 ± 0.6	17.8 ± 0.7	17.6 ± 0.5	18.3 ± 0.4	18.2 ± 0.4	18.3 ± 0.5	18.8 ± 0.6				
MCHC (%)	34.1 ± 0.4	34.0 ± 0.3	34.2 ± 0.7	34.1 ± 0.4	34.6 ± 0.4	34.6 ± 0.5	34.5 ± 0.3	34.9 ± 0.4				
Reticulocyte (%)	1.9 ± 0.3	1.9 ± 0.2	2.3 ± 0.4**	1.9 ± 0.2	1.6 ± 0.4	1.6 ± 0.3	1.6 ± 0.3	1.6 ± 0.4				
PLT (×10 ³ /μL)	939 ± 122	941 ± 114	1000 ± 83	958 ± 76	1003 ± 103	1049 ± 76	969 ± 138	990 ± 68				
PT (sec)	11.8 ± 2.1	12.3 ± 2.2	12.1 ± 1.4	12.2 ± 1.6	8.9 ± 0.2N	9.0 ± 0.4	9.0 ± 0.1	9.0 ± 0.2				
APTT (sec)	23.4 ± 2.2	23.5 ± 2.5	26.0 ± 2.0*	26.2 ± 2.2*	16.5 ± 2.1	18.1 ± 1.4	17.1 ± 1.5	18.2 ± 0.8				
Fibrinogen (mg/dL)	285 ± 43N	323 ± 103	292 ± 13	280 ± 22	214 ± 16N	205 ± 27	214 ± 11	220 ± 13				
WBC (×10 ³ /μL)	7.62 ± 1.78N	8.26 ± 1.22	7.57 ± 2.93	8.01 ± 1.47	4.48 ± 1.57N	5.69 ± 2.25	4.75 ± 1.60	4.96 ± 0.65				
Differential leukocyte ratios												
NEUT (%)	17.4 ± 4.2	21.0 ± 8.0	18.1 ± 6.9	16.3 ± 5.5	15.2 ± 3.6	17.2 ± 7.3	16.3 ± 5.6	13.9 ± 4.3				
LYMPH (%)	77.2 ± 4.2	72.7 ± 9.2	76.8 ± 6.5	77.8 ± 6.0	79.0 ± 4.1	76.1 ± 7.6	77.2 ± 6.0	80.3 ± 4.2				
MONO (%)	2.9 ± 0.8	3.5 ± 1.2	2.5 ± 0.7	3.0 ± 0.9	2.9 ± 1.0	3.2 ± 1.4	3.3 ± 1.0	3.1 ± 0.8				
EOSN (%)	1.5 ± 0.3N	1.6 ± 0.8	1.7 ± 0.6	1.8 ± 0.7	2.0 ± 0.6	2.3 ± 1.0	2.2 ± 0.6	1.7 ± 0.6				
BASO (%)	0.1 ± 0.0N	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1				
LUC (%)	0.9 ± 0.3	1.1 ± 0.6	0.8 ± 0.4	1.0 ± 0.6	0.9 ± 0.3	1.2 ± 0.6	0.9 ± 0.4	0.9 ± 0.3				

RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; PT, prothrombin time; APTT, activated partial thromboplastin time; WBC, white blood cells; NEUT, neutrophil; LYMPH, lymphocyte; MONO, monocyte; EOSN, eosinophil; BASO, basophil, LUC, large unstained cells; N, non-parametric analysis.

Mean ± SD are shown.

Significant difference from control group, *p ≤ 0.05; **p ≤ 0.01.

Table 6 Blood biochemistry data of rats treated with bamboo CNFs in 90-day repeated dose toxicity test

Sex	Male					Female						
	Dose level (mg/kg/day)		0		50	100	200	0		50	100	200
	Number of animals		10	10	10	10	10	10	10	10	10	9
Albumin (g/dL)	2.92 ± 0.16N		2.86 ± 0.29	2.80 ± 0.09	2.95 ± 0.14	3.69 ± 0.22	3.64 ± 0.33	3.51 ± 0.30	3.52 ± 0.21			
Total protein (g/dL)	5.75 ± 0.15		5.79 ± 0.25	5.72 ± 0.18	5.93 ± 0.19	6.41 ± 0.21	6.31 ± 0.42	6.11 ± 0.39	6.19 ± 0.32			
A/G (%)	1.04 ± 0.12		0.99 ± 0.15	0.96 ± 0.07	0.99 ± 0.08	1.36 ± 0.14	1.37 ± 0.16	1.35 ± 0.12	1.32 ± 0.09			
Total bilirubin (mg/dL)	0.07 ± 0.01		0.07 ± 0.01	0.07 ± 0.02	0.08 ± 0.02	0.09 ± 0.02	0.09 ± 0.03	0.09 ± 0.03	0.09 ± 0.02			
AST (U/L)	66 ± 7N		73 ± 11	69 ± 14	75 ± 20	79 ± 15	72 ± 20	68 ± 13	64 ± 11			
ALT (U/L)	28 ± 3N		32 ± 5	28 ± 6	31 ± 9	25 ± 6	25 ± 8	20 ± 4	20 ± 4			
ALP (U/L)	307 ± 74		360 ± 61	273 ± 50	296 ± 88	132 ± 27	149 ± 53	146 ± 54	151 ± 35			
Gamma-GTP (U/L)	0.6 ± 0.2		0.6 ± 0.3	0.6 ± 0.2	0.6 ± 0.3	0.9 ± 0.2	0.7 ± 0.4	1.0 ± 0.2	0.9 ± 0.2			
BUN (mg/dL)	14.8 ± 2.5		14.4 ± 1.9	15.3 ± 3.5	14.0 ± 1.8	15.9 ± 3.4	15.5 ± 3.1	15.5 ± 3.8	14.5 ± 2.5			
Creatinine (mg/dL)	0.30 ± 0.04		0.29 ± 0.03	0.31 ± 0.06	0.31 ± 0.04	0.34 ± 0.07	0.37 ± 0.07	0.37 ± 0.10	0.38 ± 0.05			
Glucose (mg/dL)	168 ± 20		171 ± 19	162 ± 18	153 ± 16	142 ± 17	147 ± 21	150 ± 17	156 ± 16			
Triglyceride (mg/dL)	71 ± 38		76 ± 27	75 ± 41	60 ± 31	27 ± 19	32 ± 19	31 ± 18	32 ± 14			
Total cholesterol (mg/dL)	67 ± 16		64 ± 10	73 ± 18	64 ± 15	77 ± 15	69 ± 17	63 ± 11	69 ± 16			
Ca (mg/dL)	9.64 ± 0.21		9.64 ± 0.34	9.58 ± 0.26	9.64 ± 0.29	9.85 ± 0.31	9.78 ± 0.35	9.75 ± 0.25	9.76 ± 0.27			
P (mg/dL)	6.10 ± 0.71		5.80 ± 0.83	5.92 ± 0.50	5.77 ± 0.63	5.02 ± 0.66	5.09 ± 0.56	5.40 ± 0.61	4.92 ± 0.96			
Na (mmol/L)	144.1 ± 0.7		144.8 ± 0.8	144.7 ± 0.5	144.8 ± 0.7*	144.0 ± 1.2	143.5 ± 0.6	143.3 ± 0.8	143.8 ± 0.9			
K (mmol/L)	4.47 ± 0.20		4.39 ± 0.13	4.53 ± 0.22	4.41 ± 0.19	4.50 ± 0.24	4.34 ± 0.22	4.48 ± 0.33	4.35 ± 0.38			
Cl (mmol/L)	106.3 ± 1.2		106.5 ± 1.1	106.8 ± 1.1	106.2 ± 0.7	107.8 ± 1.5	107.5 ± 1.6	107.6 ± 1.4	107.7 ± 0.9			

A/G, albumin/globulin ratio; AST, aspartate aminotransferase, ALT, alanine aminotransferase; ALP, alkaline phosphatase; GTP, glutamyl transpeptidase; BUN, blood urea nitrogen; N, non-parametric analysis.

Mean ± SD are shown.

Significant difference from control group, *p ≤ 0.05

Table 7 Organ weight data of rats treated with bamboo CNFs in 90-day repeated dose toxicity test

Sex	Male					Female				
	Dose level (mg/kg/day)									
	0	50	100	200		0	50	100	200	
Number of animals	10	10	10	10		10	10	10	9	
Final body weight (g)	474 ± 66	472 ± 49	516 ± 43	521 ± 41		265 ± 24	294 ± 31	288 ± 27	290 ± 19	
Brain (g)	2.09 ± 0.06	2.05 ± 0.10	2.10 ± 0.07	2.10 ± 0.12		1.90 ± 0.08N	1.93 ± 0.05	1.93 ± 0.10	1.96 ± 0.03	
Heart (g)	1.35 ± 0.16	1.39 ± 0.20	1.49 ± 0.11	1.52 ± 0.17		0.86 ± 0.04N	0.93 ± 0.11	0.98 ± 0.09*	0.91 ± 0.05	
Lungs (g)	1.44 ± 0.11	1.43 ± 0.15	1.47 ± 0.09	1.56 ± 0.12		1.04 ± 0.04N	1.15 ± 0.11	1.13 ± 0.09	1.17 ± 0.08**	
Liver (g)	11.44 ± 2.09	11.61 ± 1.31	12.97 ± 1.69	12.32 ± 1.37		6.12 ± 0.53	6.89 ± 0.87*	6.78 ± 0.65	6.80 ± 0.44	
Kidneys (g)	2.90 ± 0.29	2.83 ± 0.33	3.05 ± 0.22	3.12 ± 0.41		1.65 ± 0.14	1.83 ± 0.18	1.90 ± 0.17**	1.81 ± 0.19	
Spleen (g)	0.67 ± 0.09N	0.77 ± 0.20	0.76 ± 0.07	0.73 ± 0.09		0.44 ± 0.05	0.49 ± 0.06	0.48 ± 0.05	0.49 ± 0.03	
Adrenal glands (mg)	53 ± 9	51 ± 6	56 ± 7	59 ± 8		58 ± 10	67 ± 7	66 ± 6	65 ± 9	
Testes (g)	3.16 ± 0.30	3.16 ± 0.33	3.34 ± 0.23	3.23 ± 0.35						
Ovaries (mg)						81 ± 14	82 ± 12	86 ± 20	86 ± 10	
Thyroid glands (mg)	23 ± 4N	19 ± 4	19 ± 4	23 ± 8		13 ± 4	15 ± 5	15 ± 4	11 ± 3	
Pituitary (mg)	11 ± 2	10 ± 3	12 ± 2	13 ± 3		13 ± 3	15 ± 4	12 ± 3	14 ± 4	
Thymus (mg)	279 ± 76	313 ± 71	350 ± 55	306 ± 62		239 ± 46N	234 ± 41	245 ± 18	243 ± 82	
Prostate (mg)	1475 ± 350	1501 ± 253	1616 ± 197	1574 ± 241						
Uterus (mg)						537 ± 181	605 ± 217	537 ± 109	736 ± 299	
Salivary glands (g)	0.69 ± 0.07	0.69 ± 0.11	0.70 ± 0.09	0.73 ± 0.08		0.44 ± 0.03	0.43 ± 0.03	0.46 ± 0.05	0.46 ± 0.05	
Seminal vesicle (mg)	1446 ± 299	1592 ± 155	1515 ± 170	1627 ± 263						

N, non parametric analysis.

Mean ± SD are shown.

Significant difference from control group, * $p \leq 0.05$; ** $p \leq 0.01$.

Table 8 Summary of necropsy findings of rats treated with bamboo CNFs in 90-day repeated dose toxicity test

Sex		Male				Female			
Dose level (mg/kg/day)		0	50	100	200	0	50	100	200
Number of animals		10	10	10	10	10	10	10	9
Organ	Findings								
Hematopoietic system									
spleen	enlarged	0	1	0	0	0	0	0	
	nodule	0	1	0	0	0	0	0	0
	white patch	0	0	0	0	0	1	0	0
Respiratory system									
lungs	brown patch	2	1	0	0	0	0	0	0
nasal cavity	red patch	0	0	0	0	0	0	0	0
Digestive system									
small intestine	nodule	0	0	0	0	0	0	1	0
Urinary system									
kidneys	depression, focal	2	0	0	1	0	1	1	0
	white patch	0	1	0	0	0	0	0	0
Reproductive system									
tests	small	0	0	0	1				
epididymides	small	0	0	0	1				
uterus	dilated lumen					1	1	0	3
vagina	nodule					0	0	0	1
Endocrine system									
pituitary gland	cyst	0	1	0	0	0	0	0	0
thyroid glands	small	0	0	0	0	0	1	0	0
adrenal glands	brown patch	0	0	1	0	0	0	0	0

ness. In addition, in the test for water-soluble substances, the measured mass of the filtrate that passed through the filter paper for quantitative analysis (Type 5 C) exhibited higher residue in the test specimen compared with the specified value. For this reason, the bamboo CNFs were more nano-sized than were fine fibrous cellulose, which contains many fine fibers that pass through the filter paper. Therefore, bamboo CNFs do not fall into the category of fine fibrous cellulose that is specified in food additives.

None of the following tests, conducted to investigate the safety of bamboo CNFs, found any notable problems under the test conditions: bacterial reverse mutation test, *in vitro* mouse lymphoma TK assay, and an *in vitro* micronucleus test using Chinese hamster cells. In addition, no deaths were observed in any of the treatment groups throughout the administration period in a repeated dose 90-day oral toxicity study using rats. No effects on administration of the test substance were observed with respect to general conditions, urinalysis, or ophthal-

mologic examination. There were no significant bamboo CNF-related adverse effects on hematological examination, blood biochemical examination, or pathological examination (organ weight measurement, macroscopic observation of organs/tissues, and histopathological examinations) performed at the end of the administration period. Food intake tended to be high throughout the administration period in the male 100 and 200 mg/kg/day groups and in all female test substance-administered groups. Along with the increased food intake, changes in body weight also tended to be higher, but the change was mild and considered an increased appetite, so it was judged to have no toxicological significance. Furthermore, hematological examination also revealed a statistically significant prolongation of APTT in the male 100 and 200 mg/kg/day groups, but the degree of change was negligible and considered to have no toxicological significance. Based on the above results obtained in the 90-day repeated oral toxicity study of the bamboo CNFs in rats, no toxicity was observed from substance

Table 9 Summary of histopathological findings of rats treated with bamboo CNFs in 90-day repeated dose toxicity test

Sex		Male				Female			
Dose level (mg/kg/day)		0	50	100	200	0	50	100	200
Number of animals		10	10	10	10	10	10	10	9
Organ	Findings								
Cardiovascular system		1	1	0	2	1	1	0	1
heart	cardiomyopathy								
Hematopoietic system									
spleen	necrosis	0	1	0	0	0	0	0	0
	capsulitis	0	0	0	0	0	1	0	0
lymph nodes	capsulitis	0	0	0	0	1	0	0	0
thymus	cyst	0	0	0	0	2	1	3	2
Respiratory system									
lungs	hemorrhage	0	1	0	0	0	0	0	0
	metaplasia, osseous	0	1	1	1	1	0	0	1
	aggregation, macrophage	5	4	3	4	2	3	1	1
	granuloma, foreign body	0	2	0	0	0	0	0	0
	inflammatory change	2	0	0	0	0	1	0	0
nasal cavity	mineralization	9	8	9	9	5	5	6	6
Digestive system									
pancreas (exocrine)	nodule	3	2	1	3	3	2	0	3
jejeum	cyst	0	0	0	0	0	0	1	0
liver	fatty change, hepatocyte	4	5	7	5	7	4	8	6
	arteritis	1	0	0	0	0	0	0	0
	microgranuloma	8	8	9	9	10	8	8	8
mandibular glands	infiltration, inflammatory cell	0	1	1	0	0	0	0	0
sublingual glands	infiltration, inflammatory cell	0	0	0	0	0	0	1	0
Urinary system									
kidneys	cast, hyaline	1	1	0	2	0	0	0	0
	cyst	0	1	1	1	0	0	0	0
	hyaline droplet	4	1	1	2	0	0	0	0
	mineralization	3	3	4	2	5	4	7	5
	regeneration, tubule	6	5	3	6	2	2	0	3
	infiltration, inflammatory cell	0	0	0	0	0	1	1	0
	inflammatory change	1	0	0	1	0	1	2	1
urinary bladder	infiltration, inflammatory cell	0	0	1	0	0	0	0	0
Reproductive system									
testes	atrophy, seminiferous tubule	0	0	0	1	0	0	0	0
epididymides	infiltration, inflammatory cell	5	4	6	4				
	decrease, sperm	0	0	0	1				
prostate	inflammatory change	9	7	7	8				
ovaries	cyst					1	1	1	0
Uterus	dilatation, lumen					2	1	0	3
vagina	cyst					0	0	0	1
Endocrine system									
pituitary gland	cyst	0	1	1	0	0	1	0	0
	craniopharyngeal duct, remnant	0	1	0	0	0	0	0	0
parathyroid glands	fibrosis	1	0	0	0	0*	1	0	0
pancreas (endocrine)	deposit, pigment	1	1	1	0	0	0	0	0
Special sense system									
eyes	disarrangement, retinal	0	0	0	0	0	2	0	0
Harderian glands	Inflammatory change	2	0	1	1	3	1	0	3

*Number of animals examined microscopically at this site was 9.

administration in both male and female rats, and the NOAEL (no observed adverse effect level) under these test conditions was determined to be 200 mg/kg/day or more.

Microfibrinous cellulose, which is made by refining cellulose, improves the physical characteristics of cellulose. The changes in the functionality of foods resulting from its addition are being investigated. According to Nakamura *et al.*,⁷⁻⁹⁾ when the effect of microfibrinous cellulose in rats was examined by feeding them with pectin and sugar, the same effect was obtained as dietary fiber, that is, an increase of wet weight, dry weight, water retention of feces, and the reduction of gastrointestinal transit time. In addition, the analysis of rat feces fed with microfibrinous cellulose revealed a decrease in the apparent absorption rate for zinc and iron⁸⁾. Adsorption of zinc and iron may be inhibited or promoted depending on the physicochemical properties of the dietary fiber to be added and its intake¹⁰⁾. Therefore, a detailed examination is required for each case. To clarify the effects of the addition of CNFs, which is made by nano-sizing cellulose, Liu and Kong¹¹⁾ showed that CNFs suppress the amount of free fatty acids produced in the process of decomposing milk fat and increase the adsorptive property of iron *in vitro* compared to cellulose. Additionally, DeLoid *et al.*¹²⁾ reported that nano-sized fine fibers of CNFs prevented fat digestion and absorption more than that of cellulose. The amount of CNF used in food is expected to increase in the future, because CNFs may improve the texture and physical properties of conventional dietary fiber. They may also improve the properties of food materials to make them easier to use. It is important to elucidate the functions related to food use of CNFs and to accumulate more information related to safety, but not limited to bamboo CNF.

Acknowledgments

This research was carried out as part of the Bio-oriented Technology Research Advancement Institution 2014-2016 innovative technology creation promotion project (interdisciplinary joint research). We would like to express our gratitude to everyone involved. We also express our sincere gratitude to Mr. Masanobu Nojiri and other members of FFPRI, Microbial Engineering Laboratory, Polysaccharide Chemistry Laboratory, and Wood Chemistry Laboratory for their support.

References

- 1) Forestry Agency, Ministry of Agriculture, Forestry and Fisheries, Japan : <https://www.rinya.maff.go.jp/j/keikaku/genkyou/h29/attach/pdf/3-7.pdf> (2017)
- 2) Shimokawa T., *et al.* : Bull. Forestry Forest Products Res. Inst. 19, 341-348 (2020)
- 3) Gómez H. C., *et al.* : Food Hydrocolloids 57, 178-186 (2016)
- 4) Bouwmeester H., *et al.* : Trend Food Sci. Technol. 40, 200-210 (2014)
- 5) EFSA Scientific Committee : EFSA J. 9, 2140 (2011)
- 6) Consumer Affairs Agency, Ministry of Health, Labor and Welfare, Japan : 8th Edition Japan's Specification and Standards for Food Additives, https://www.mhlw.go.jp/seisakunitsuite/bunya/kenkou_iryou/shokuhin/syokuten/dl/8e03.pdf.
- 7) Nakamura K., *et al.* : J. Jpn. Soc. Nutr. Food Sci. 41, 185-189 (1988)
- 8) Nakamura K., *et al.* : J. Jpn. Soc. Nutr. Food Sci. 41, 191-196 (1988)
- 9) Nakamura K., *et al.* : J. Agric. Sci. Tokyo Univ. Agric. 33, 262-269 (1989)
- 10) Japanese Association for Dietary Fiber Research : Dietary Fiber, Daiichi shuppan, 2008, 182-183
- 11) Liu L. and Kong F. : Int. J. Biol. Macromol. 137, 1278-1285 (2019)
- 12) Deloid G. M., *et al.* : ACS Nano 12, 6469-6479 (2018)