Safety Evaluation of Cellulose Nanofibers produced from Soda-anthraquinone Sugi pulp by Enzymatic Treatment and Wet milling

Tomoko Shimokawa^{*2}, Kengo Magara, Masanobu Nojiri and Noriko Hayashi

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

Tomoko Shimokawa

Kengo Magara

Masanobu Nojiri

Noriko Hayashi

Abstract

Cellulose nanofiber (CNF) is a promising new material used in a wide range of industries. The application of soda-anthraquinone cooking of sugi (Japanese cedar) and subsequent nanofibrillation of the enzymatically treated pulp by wet milling enabled us to establish a simple integrated manufacturing process to obtain sugi-CNFs. However, there are concerns that the sugi CNF might be mutagenic or cause skin irritations; therefore, we tested these hypotheses. Acute oral toxicity test using mice showed no abnormality, with an LD₅₀ value of over 2,000 mg/kg. Also, the sugi CNF was found to be non-mutagenic in the bacterial reverse mutation test. In addition, no anomalies were detected in the *in vitro* chromosomal aberration test using Chinese hamster lung fibroblast cells. Moreover, the CNF treatment of rabbits in dermal irritation or corrosion tests did not produce severe dermal irritation or corrosion or distinctive signs of toxicity. Erythema was observed in the site of 5wt% CNF application in all the tested animals one hour after the CNF application ended; however, erythema disappeared by the 48th hour. As a result, the sugi CNF was evaluated to be in the non-irritating category. According to the results from the guinea-pig maximization test, which predicts skin sensitization, the effect of the sugi CNF was classified as the weakest grade. In conclusion, the sugi CNF is unlikely to cause mutagenicity in organisms. In addition, the level of skin irritation caused by the sugi CNF is within the normal range of handling.

Keywords : cellulose nanofiber, sugi, chromosomal aberration test, acute dermal irritation/corrosion test, maximization test

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

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

1. Introduction

Cellulose nano-fiber (CNF) is a new material made from pulp, has been used in kinds of commodities and is being available in markets. Its quality is dependent on wood species and processes applied for manufacturing, and users would ask different qualities of their own. So, CNF of specialty grades will be in demand along with that of commodity grade in the future. Therefore, as a process feasible for newcomers other than paper companies, we chose the integrated process of soda-anthraquinone cooking followed by enzymatic treatment and then by wet milling. The resulting CNF were marketed as under paint for wood products¹⁻³⁾.

When CNF is manufactured by a batch process of small volume, there will be more chance of directly contacting CNF itself, compared to a case of large continuous process. Though CNF that is disintegrated to nano-level size is not a new product, the data on its safety are too lacking to develop its applications as an industrial material. Therefore, we examined its safety by various evaluations as a part of our project of developing the integrated manufacturing process.

Firstly, we did acute oral toxicity test and got LD₅₀, the value over which more than a half of the tested animals would die by oral administration of single dose. Secondly, we did bacterial reverse mutation test and chromosomal aberration test using mammalian cultivated cells, defined in the Act on the Regulation of Manufacture and Evaluation of Chemical Substances (Chemical Substance Control Law) as a screening test for toxicity. Though bacterial reverse mutation test is reported to be not suitable for nano-materials⁴⁾, it is a common elemental toxicity test, and we thought the test would help accumulate more data and verify its extensive safety. Regarding to chromosomal aberration test using mammalian cultivated cells, it was applied to carbon nano-tube⁵⁾, and would be needed for CNF, one of nano-materials, as well.

Furthermore, to verify the effect on skin, we did dermal irritation/corrosion tests on rabbits and skin sensitization test on guinea pigs. We report the result of those tests as follows.

2. Experiments

2.1 Preparation of pulp by soda-anthraquinone cooking

150 g (oven dry base) of air-dried sugi (*Cryptomeria japonica*) chips were put into a 2 L capacity static stainless autoclave (Nitro Autoclave, Type NAC13). 900 mL of cooking liquor that consisted of 26% sodium hydroxide and 0.1% anthraquinone compound (1,4-dihydro-9,10-

dihydroxyanthracene, Kawasaki Kasei Chemicals LTD.) per dry chip weight was placed to the autoclave. It was heated up to 170°C in 90 minutes and kept at that temperature for 120 minutes. At the end of cooking, its pressure was relieved to a blow tank of 20 L to be atmospheric. After removing black liquor, the chips were disintegrated by a disintegrator (Kumagai Riki, No. 2530), and screened by a flat screen (Kumagai Riki, No. 2625, slit width : 8/1,000 inches).

2.2 Bleaching

The oxygen-bleaching was carried out in a pulp mixer designed for medium consistency (Quantum, Mk5). A 0.2% aqueous sodium hydroxide solution was added to 120 g (oven dry base) of sugi pulp prepared by soda-anthraquinone cooking to make a pulp slurry having a total volume of 1.2 L. The pulp slurry was charged into a 2 L mixing bowl and the top cover was closed under 1.0 MPa. The temperature was raised up to 90°C with mixing at 600 rpm for 15 seconds in every minute. Then, oxygen was added at the constant pressure of 0.6 MPa, and it was mixed at 2,000 rpm for 20 seconds. After the oxygen charge, the bleaching was carried out for 60 minutes by mixing at 600 rpm in every minute. After bleaching, oxygen was purged and the pulp was washed with water on 300 mesh screen until neutral pH.

For ozone bleaching, a pulp mixer for medium consistency was also used as in oxygen bleaching. 12 g (oven dry base) of pulp was diluted with water to prepare a 1.2 L slurry, and the pH was adjusted to 2 with phosphoric acid. Then, the pulp slurry was charged into a 2 L mixing bowl and the top cover was closed. 5 L of oxygen gas containing ozone was fed to the mixing bowl at the pressure of 0.7 MPa under room temperature with mixing at 2,000 rpm for 20 seconds, followed by mixing at 600 rpm for 30 seconds. After the bleaching, the pressure was discharged. The amount of ozone supplied in the batch was 0.4% against 120 g of pulp. The procedure was repeated until the amount of ozone supplied became 2%. After bleaching, the pulp was washed with water on 300 mesh screen until neutral pH.

Sodium hydroxide solution was added to prepare the 10% ozone-bleached pulp slurry. The amount of sodium hydroxide was 2% against the pulp. The pulp slurry was sealed in a polyethylene bag and kept for one hour at 70°C for alkali extraction. Then, it was washed with water on 300 mesh screen until it became neutral in pH.

2.3 Enzymatic treatment and wet milling

The bleached pulp was enzymatically treated as follows. The 1.6% pulp slurry was prepared with ion-exchanged water, to which endoglucanase (Family 45, a sample product) was added at the rate of 1 mg (equivalent to protein) per 1 g (oven dried) of the pulp.

Fig. 1 Electron micrograph of microfibrillated sugi pulp

The pH of the slurry was adjusted to 4.5, and then the reaction was carried out at 50°C for 6 hours. The protein content of the enzyme sample was determined by quick start Bradford (BioRad) using bovine gamma globulin as a standard. The enzymatic reaction was stopped by thermal treatment of 110°C. Then, the reactant was subjected to a bead mill of circulation type (LMZ2, Ashizawa Finetech Ltd.) equipped with zirconia beads (ϕ 1 mm). The peripheral velocity of the bead was 10 m/sec and the milling was conducted to make Dv 90 below 10 nm, measured by a particle size analyzer (LA-960, Horiba). Dv 90 was defined as the diameter at which 90% of the population counted by volume lies below this value⁶⁾.

The resulting CNF was observed with optical microscope as well as with electron scanning microscope (TEM, JEM-2000EX, JEOL). It was verified that the milled products contained some branched CNF of which width was 3–100 nm and of which length was within 5 μm ^{1,7)} (Fig. 1).

2.4 Acute oral toxicity test

The acute oral toxicity test using mice was conducted with reference to the OECD TG420 (2001) at Japan Food Research Laboratories. The CNF specimen was condensed to 5 wt% by heating under 70°C and sterilized in autoclave. As no data were available for the toxicity, the maximum dosage of the CNF was designed to be 2,000 mg/kg. So, the levels of dosage were determined to be 2,000, 1,000, 300 and 50 mg/kg respectively. Five male and five female ICR mice per every dose group, five-week-old after a week of preliminary breeding, were orally administrated as follows. The CNF specimens, diluted with injection water to the administration rates of 1,000, 500, 150 and 25 mg/kg respectively, were forcibly orally administrated using gastric tube twice in the first day with an interval of about 2 hours. The animals were then housed in a controlled room under lightning 12 hours a day at 23°C. All animals were

observed for 14 days after administration, and subjected to necropsy.

2.5 Bacterial reverse mutation test

Salmonella typhimurium (histidine auxotroph : TA98, TA100, TA1535, TA1537) and *Escherichia coli* (tryptophan auxotroph : WP2uvrA) (Japan Bioassay Research Center) were used for the test. Referring to the standards for mutagenicity test with bacteria published by the Ministry of Labor (Notification No. 77, September 1, 1988) and its partial revision (Notification No. 67, June 2, 1997), and the procedures and evaluations for mutagenicity test with bacteria (Notification by Chemical Research Division, Safety and Health Department, Standard Bureau, Ministry of Labor, February 8, 1999), two cases were performed in the presence (+S9 mix) and absence (–S9 mix) of activation using rat-liver homogenate (S9, Kikkoman Biochemifa Co.).

A dose-finding test (6.86–5,000 $\mu\text{g}/\text{plate}$) and a primary test (312.5–5,000 $\mu\text{g}/\text{plate}$) were carried out by the preincubation method. The CNF specimen was condensed under 70°C to 5 wt%, and sterilized in autoclave. By reason of the low fluidity of the specimen to carry out the tests, the maximum concentration practicable was checked to be 1.25%. So, it was diluted with injection water and homogenized by ultrasonic treatment. As the maximum dose recommended in the guideline was 5,000 $\mu\text{g}/\text{plate}$, the dose in the test substance group was set to 0.4 mL/plate, and the dose in the negative control group was also 0.4 mL/plate. In the positive control group, the usual dose of 0.1 mL/plate was used, and to get better comparison, the same dose volume (0.1 mL/plate) was additionally applied to the negative control.

The test procedure was as follows. A solution, anyone of the negative controls, the test specimens and the positive controls, was taken into a test tube, to which 0.1 mol/L Na-phosphate buffer (pH 7.4) or S9mix (0.5 mL) and bacterial inoculate (0.1 mL) were added. The tube was shake-cultured at 37° C for 20 minutes, to which 2 mL of top agar (0.6% soft agar containing 0.5% sodium chloride, with the addition of L-histidine for *S. typhimurium* and with L-tryptophan for *E. coli* cases) was blended. After mixing, the content was poured onto a minimum glucose agar plate, and incubated for 48 hours at 37°C. Then, the number of colonies was counted. The test work was done at SRD Biology Center Co., Ltd.

2.6 Chromosomal aberration test using mammalian cultivated cells

The test was carried out according to the notification by the Ministry of Health, Labour and Welfare (No. 652, September 29, 1997) entitled “The amendment of the

standards for chromosomal aberration test using mammalian cultivated cells and its report forms” and the notifications by the Ministry of Health, Labour and Welfare the Ministry of Economy, Trade and Industry and the Ministry of The Environment (No. 0331-7, 2011, March 31, No.5, March 29, 2011, and No. 110331009 respectively) entitled “On the test procedure of new chemical compounds” and their partial amendments (No. 0102-1, April 2, 2012, No. 2, March 28, 2012, and No. 120402001, respectively). The test was done at SRD Biology Center Co., Ltd. A CNF specimen was condensed under 70°C to 5 wt%, and sterilized in autoclave. Fibroblast cell from Chinese hamster lung (CHL/IU, National Institute of Biomedical Innovation) was used. The four kinds of testing conditions were designed, two short-time treatments, one metabolically activated with rat-liver homogenate (+S9mix, Kikkoman Biochemifa) and the other without activation (−S9mix), and two continuous treatments, 24 hours and 48 hours. The cell was incubated in MEM (Minimum Essential Medium) liquid medium (GIBCO), to which 10% Bovine serum was added, in a CO₂ incubator of which CO₂ concentration was 5% at 37°C under humidified. After pre-incubation followed by trypsin treatment, suspended cells were counted with a use of hemocytometer. Then, the cell suspension was diluted with the medium to make its cell density to be 1×10^4 cells/mL, which would be used in cell growth inhibition test and chromosomal aberration test. In the cell proliferation test, in which a number of cells was an indicator, the guideline recommended 5 mg/mL of the test substrate as the maximum. Thus, the dosage of CNF specimen was determined to be six levels, 5 (the largest), 1.667, 0.185, 0.062, 0.021 and 0.007 mg/mL respectively, by diluting with the medium. In the case of short-time treatment with +S9mix, the final concentration of S9 was 5%. Though no retardation in cell growth was observed in any dose levels, some flocculation appeared over the dose of 0.556 mg/mL. So, in chromosomal aberration test, three dose levels, 0.6, 0.3 and 0.15 mg/mL, were selected that covered the transition zone from flocculation to non-flocculation. For the negative control, the medium was used. Regarding to the positive control, mitomycin C (MMC) was used for test groups of −S9mix and benzo [a] pyrene (B [a] P) was used for a test substance group of +S9mix.

In the short-time treatment, the incubation was carried out for 3 days, and then the whole medium was discarded. The CNF specimen solution was added to the residue which was, then, incubated for 6 hours, one set of tests being with S9mix and the other set without S9mix. Then, the Petri dish was washed with physiological saline of 37°C and to which new culture

medium was added and incubated for another 18 hours. In the continuous treatment, after 3 days-pre-incubation, incubations lasted, one for 24 hours and the other for 48 hours, to get specimens of chromosome analysis.

Colcemid solution (Wako Pure Chemical Industries) was added to each specimen, 2 hours prior to be finished, to stop cell division. After the cultivation, cells were made floated with trypsin treatment and recovered. The cells were swollen with 75 mM KCL solution, solidified with Carnoy's fluid and stained with Giemsa staining solution for observation. One hundred metaphases per plate were observed by differential interference microscope. Structural and numeric chromosomal aberrated cells were counted. Regarding to the structural aberrancy, chromatid break, chromatid exchange and other damages like fragmentation were examined. The criterion of chromatid gap was non-staining region of a single chromatid in which there is minimal misalignment of the chromatid. Regarding to the numeric aberrancy, polyploid and endoreduplication were separately counted.

2.7 Dermal irritation or corrosion test using rabbits

According to OECD TG 404 (2021), dermal irritation or corrosion test, using rabbits was done at Japan Food Research Laboratories. One CNF specimen was prepared by diluting a 1.6 wt% CNF suspension with distilled water to 1 wt%, and the other was concentrated to 5 wt% under 70°C. They were sterilized in autoclave respectively. Three Japanese white male rabbits (Kitayama Labes) were used after verification that there were no abnormalities during preliminary breeding of more than a week. They were reared at a room temperature of 23°C, under lightening of 12 hours per day. Solid feed for rabbit and guinea pig (LRC4, Oriental Yeast) was control-fed and tap water was free intake. Hair on a part of back trunk of the body was sheared 24 hours ahead of the test. 6 spots, 2×3 cm/spot, were selected in the sheared area, three of which were scar marked or injured in a parallel cross shape with injection needle of 18 gauge, deep down to a cornified layer, but not so deep to derma (injured skin). The other three were unscarred or intact (intact skin). A specimen solution (0.05 mL) was applied in an open condition to one of the three spots, injured as well as intact. After 24 hours, the spots for 1 wt% specimen were cleaned with injection water, and the spots for 5 wt% specimen were cleaned with cleansing agent, soap and mild water so that residue of the specimen was removed. The spots were observed in 1 hour, 24 hours, 48 hours and 72 hours after the cleaning respectively, and resulting skin reactions were evaluated with erythema and crust as a first parameter and edema as a second parameter. For

erythema and crust, scores were graded as follows. No erythema : 0, light erythema : 1, clear erythema : 2, medium to high erythema : 3, severe erythema and crust : 4. For edema, score rates were as follows. No edema : 0, very slight edema : 1, light edema : 2, medium edema (1 mm of protuberance), high edema (more than 1 mm of protuberance and injured area) : 4. According to ISO 10993-10 Biological evaluation of medical devices-Part 10 (2010), the primary irritation index (P.I.I.) was calculated as follows. The scores of the intact and the injured for 24 hours, 48 hours and 72 hours were summed up. Then, the total was divided by 6. With regard to rabbit, P.I.I. is categorized as follows. No irritation : 0-0.4, light irritation : 0.5-1.9, medium irritation : 2.0-4.9, strong irritation : 5.0-8.0

2.8 Skin sensitization test using guinea pigs

Skin sensitization test (maximization test) using guinea pigs was done at SRD Biology Center Co., Ltd in reference to OECD TG 406 (1992). The CNF suspension prepared at 1.6 wt% consistency was diluted to 1.0 wt% with distilled water, and sterilized in autoclave. Injection water was used for diluting the specimens and for the negative control. The positive control, as recommended in the guide line, was 2,4-dinitrobenzine (DNCB) dissolved in 80% ethanol (for induction), and one dissolved in acetone (for challenge test). Female guinea pigs aged 5 weeks (Slc : Hartley, Japan SLC) were preliminary bred for 5 days, and were verified to be normal, before grouping. Five individuals were distributed to the positive control group, 5 to the negative control group, and 10 to the test substance group. The test animals were reared in a room temperature of 20°C under lightening of 12 hours a day. Solid feed (CG-7, CLEA Japan) was free intake, and tap water was also free intake. 24 hours before induction, an animal was sheared and 6 test spots (2×4 cm each) were determined, taking a center line of the back of a test animal as a symmetry axis, three spot sites in each side of the center line. The volume of intradermal injection was 0.1 mL. As for the negative control, Freund's Complete Adjuvant (FCA) emulsified with injection water at the rate of one to one (abbreviated as injection water-FCA) was for four spots and injection water only for two spots. As for the test substrate group, injection water-FCA, test specimen (100%) and test specimen (50%)-injection water were injected, two sites for each. As for the positive control, injection water-FCA, 0.1% DNCB/80% ethanol solution, and 0.1% DNCB-FCA were administered, two sites for each.

On the 6th day after the first injection, the site was sheared again on which sodium lauryl sulfate (10%)/vaseline ointment (0.5 mL) was spread open as a

prophlogistic agent. On the 7th day after the first injection, the prophlogistic agent was removed with mild water. 0.2 mL of solution, injection water for the negative control, 100% test specimen for the test substrate group and 1.0% DNCB/80% ethanol for the positive control respectively, was spread on a patch of lint cloth, which was pasted on each site as the secondary induction, and wrapped with adhesive extension bandage for 48 hours, and then removed. On the 13th day after the second induction, the right ventral part of the animal was sheared, of which area was 5×5 cm, as an area for challenge test. 0.1 mL of solution, 100% test specimen for the negative control and the subject group, and 0.1% DNCB/acetone for the positive control respectively, was spread on a patch of lint cloth, and was pasted on the challenge test site in a closed condition and kept for 24 hours, and then removed.

In 24 and 48 hours after removing the challenge patch, its dermal response was scored according to the standard by Magnusson and Kligman⁸⁾. The result of the test substrate group was compared to that of the negative control group. The criterion of the score was as follows. No change by the naked eye : 0, scattered or frequent erythema : 1, diffuse erythema : 2, strong erythema and edema : 3. The sensitization rate was calculated as a ratio of a number of animals with positive response against a number of animals observed. The maximization grade was, according to Magnusson et.al.⁸⁾ as follows. The sensitization rate from 0 to 8% : weak, 9-28% : mild, 29-64% : moderate, 65-80% : strong and 81-100% : extreme.

Among the safety tests mentioned above, those using animals complied the guideline entitled "Guideline for conducting animal experiment etc. in Forestry and Forest Products Research Institute" and an ethic guideline of each testing institution on doing experiment using animals.

3. Result

The result of the acute oral toxicity test is in Table 1. There was no fatal case among animals administered with different dosages, regardless of male or female, and abnormality was neither observed. Regarding to the body weight in 7 and 14 days after administration, there was no difference among the groups, regardless of sex. The necropsy of the tested animals after observation did not show any abnormality. It was concluded that LD₅₀ of sugi CNF was more than 2,000 mg/kg based on its real amount.

Regarding to the bacterial reverse mutation test using *S. typhimurium* and *E. coli* that could evaluate mutagenesis on genes, we determined dosages by preliminary test. Then, each bacterium was treated with sugi CNF in the

Table 1 Changes in body weight of mice treated once orally with sugi cellulose nanofiber

Sex	Dose (mg/kg)	Days after administration (g)		
		0 ^a	7	14
Male	2000	32.0 ± 1.4	37.6 ± 2.1	41.1 ± 2.7
	1000	32.2 ± 1.4	36.5 ± 2.0	39.9 ± 3.1
	300	32.1 ± 1.3	37.0 ± 2.0	39.8 ± 2.1
	50	32.1 ± 1.4	37.4 ± 1.7	40.4 ± 1.6
	0	32.1 ± 1.2	37.4 ± 1.1	40.0 ± 1.4
Female	2000	25.9 ± 2.0	29.1 ± 2.4	32.0 ± 2.6
	1000	25.7 ± 2.0	27.6 ± 1.5	30.5 ± 1.6
	300	25.9 ± 2.3	28.7 ± 1.7	32.1 ± 2.5
	50	25.7 ± 2.4	28.8 ± 3.1	30.9 ± 3.8
	0	26.1 ± 2.6	29.5 ± 3.5	32.6 ± 4.3

Note: The body weight of the mice at each treatment and time point is the average of five animals ± SD (g).

^aBefore administration.

Table 2 The effect of sugi cellulose nanofiber on the reverse mutation in bacteria

Substance	Dose (μg/plate)	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	33	22	134	109	13	9	11	5	26	22
	0.4 ml/plate	33	18	135	105	12	9	10	4	34	22
Sugi CNF	312.5	43	24	133	111	9	12	11	3	32	18
	625	34	22	138	102	14	10	10	5	33	26
	1250	31	13	141	109	10	7	12	3	33	26
	2500	40	21	129	121	14	9	13	5	27	24
	5000	32	22	135	101	14	8	9	5	37	22
AF-2	0.01				401						148
	0.1		351								
2-AA	0.5	402									
	1			1228							
	2					250		236			
	10									1137	
9-AA	80								408		
NaN ₃	0.5										

The negative control was sterile water for injection.

AF-2, 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide; 2-AA, 2-Aminoanthracene; 9-AA, 9-Aminoaridine.

dose range of 6.86–5,000 μg/plate. The number of revertant did not show any rise, compared to those of the negative control group (0.4 mL/plate) with any bacteria, whether they were metabolically activated or not (Table 2). The volume of the dose was increased to 0.4 mL/plate in this test from 0.1 mL/plate that was of normal routine. The effect of this increased volume was checked with the negative control by repeating test with the two different volumes. As there was no definite difference between the two, the volume increase would not affect the result. On the other hand, the number of revertant of the positive control for each bacterium clearly increased, compared to that of the negative control. The result was reproducibly verified both in the preliminary test and in the main test. As a result, the gene mutagenesis of sugi CNF under the test conditions was judged to be negative.

Chinese hamster lung fibroblast cell (CHL/TU cell) was used for chromosomal aberration test. The test

consisted of two short-time treatments, with S9 and without S9, and two continuous treatments, one for 24 hours and the other for 48 hours. The result is in Table 3. In the test substance groups, the number of structurally aberrant cells and the number of polyploid cells were the same level to those of the negative control. On the other hand, those of the positive control were clearly larger than those of the negative control. Based on the above results, the chromosomal aberration inducibility of sugi CNF under the test conditions was judged to be negative.

CNF specimens, 1 wt% and 5 wt%, were pasted on two types of skin surfaces, intact and injured, of a rabbit, three rabbits for each CNF respectively. For 1 wt% CNF, very light erythema was observed in two rabbits in one hour after the specimen was removed, which disappeared in 24 hours (Table 4). For 5 wt% CNF, light to distinct levels of erythema were observed in all rabbits in one hour after the specimen removed, which

Table 3 Chromosome analysis of Chinese hamster lung fibroblast cells treated with sugi cellulose nanofiber

Substance	Dose (mg/ml)	S9 mix ^a	Time ^b (h)	Number of structurally aberrant cells					Total abnormal cells (%)	Number of gap (%)	Number of polyploid cells (%)	Cell proliferation rate (%)
				ctb	cte	csb	cse	frg				
Negative control	0	–	6-18	1	0	0	0	0	1 (0.5)	1 (0.5)	0 (0.0)	100
Sugi CNF	0.15	–	6-18	0	0	0	0	0	0 (0.0)	0 (0.0)	0 (0.0)	99
	0.30	–	6-18	0	0	0	0	0	0 (0.0)	0 (0.0)	0 (0.0)	105
	0.60	–	6-18	1	0	0	0	0	1 (0.5)	1 (0.5)	0 (0.0)	97
MMC	0.0001	–	6-18	17	37	0	0	0	48 (24.0)	4 (2.0)	0 (0.0)	NT
Negative control	0	+	6-18	0	0	0	0	0	0 (0.0)	0 (0.0)	0 (0.0)	100
Sugi CNF	0.15	+	6-18	1	1	0	0	0	1 (0.5)	0 (0.0)	2 (1.0)	99
	0.30	+	6-18	0	1	0	0	0	1 (0.5)	0 (0.0)	2 (1.0)	93
	0.60	+	6-18	0	0	0	1	0	1 (0.5)	0 (0.0)	0 (0.0)	109
B(a)P	0.02	+	6-18	15	85	0	0	0	87 (43.5)	1 (0.5)	1 (0.5)	NT
Negative control	0	–	24-0	0	0	0	0	0	0 (0.0)	0 (0.0)	0 (0.0)	100
Sugi CNF	0.15	–	24-0	0	0	0	0	0	0 (0.0)	0 (0.0)	0 (0.0)	99
	0.30	–	24-0	0	0	0	0	0	0 (0.0)	0 (0.0)	0 (0.0)	95
	0.60	–	24-0	0	0	0	0	0	0 (0.0)	0 (0.0)	0 (0.0)	97
MMC	0.0001	–	24-0	23	51	0	0	0	62 (31.0)	1 (0.5)	1 (0.5)	NT
Negative control	0	–	48-0	1	0	0	0	0	1 (0.5)	0 (0.0)	1 (0.5)	100
Sugi CNF	0.15	–	48-0	1	0	0	0	0	1 (0.5)	0 (0.0)	0 (0.0)	90
	0.30	–	48-0	0	1	0	0	0	1 (0.5)	0 (0.0)	0 (0.0)	99
	0.60	–	48-0	0	0	0	0	0	0 (0.0)	0 (0.0)	0 (0.0)	90
MMC	0.0001	–	48-0	30	66	0	2	1	79 (39.5)	6 (3.0)	1 (0.5)	NT

^aS9 mix was used for metabolic activation.

^bTime shows treatment time-recovery time.

The negative control was medium.

MMC, mitomycin C; B(a)P, benzo(a)pyrene.; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; frg, fragmentation; gap, an achromatic lesion smaller than the width of one chromatid; NT, not tested.

Table 4 Acute dermal irritation and corrosion study of sugi cellulose nanofiber in rabbits

Dose (wt%)	Time after removal of patches (h)	Score of erythema/edema formation					
		Animal ID 1		Animal ID 2		Animal ID 3	
		intact	injured	intact	injured	intact	injured
1	1	1/0	1/0	1/0	1/0	0/0	0/0
	24	0/0	0/0	0/0	0/0	0/0	0/0
	48	0/0	0/0	0/0	0/0	0/0	0/0
	72	0/0	0/0	0/0	0/0	0/0	0/0
	P.I.I.*	0.0					
5	1	2/0	2/0	2/0	2/0	1/0	1/0
	24	1/0	1/0	1/0	0/0	1/0	1/0
	48	0/0	0/0	0/0	0/0	0/0	0/0
	72	0/0	0/0	0/0	0/0	0/0	0/0
	P.I.I.*	0.3					

The dermal responses were scored into 5 levels; score 0, no abnormal detected; score 1, weak; score 2, mild; score 3, moderate; score 4, strong.

*P.I.I., Average of calculated values ((scores of erythema of intact skin and injured skin after 24h + scores after 48h + scores after 72h)/6) for each test animal.

Table 5 Skin sensitization study of sugi cellulose nanofiber in guinea pigs

Substance for induction		Substance for challenge	Number of animals	Hours after the end of challenge	Average sensitization score ^a	Sensitization rate (%) ^b	Maximization grade ^c
Intradermal injection	Patch applicataion						
Water for injection	Water for injection	100% sugi CNF (1wt%)	5	24 48	0.0 0.0	NT	NT
100% sugi CNF (1wt%)	100% sugi CNF (1wt%)	100% sugi CNF (1wt%)	10	24 48	0.0 0.0	0	I weak
0.1% DNCB	1.0% DNCB	0.1% DNCB	5	24 48	3.0 3.0	100	V Extreme

^aThe dermal responses were scored on a 4-point scale from 0 to 3 according to Magnusson and Kligman (1969).

^bThe sensitization rate is calculated as (number of animals with positive response/number of animals observed)×100.

^cMaximization grades (I-V) were assigned according to Magnusson and Kligman (1969).

DNCB, 2,4-Dinitrochlorobenzene; NT, not tested.

disappeared in 48 hours. According to ISO 10993-10 Biological evaluation of medical devices-Part 10 (2010), the primary irritation index (P.I.I.) was 0 for 1 wt% CNF and 0.3 for 5 wt% CNF. Accordingly, in the dermal irritation or corrosion test using rabbits, the CNF specimens at each concentration were evaluated to be in the category of “none-irrelative” under the test condition in this study.

In the skin sensitization test examined by the maximization method using female Hartley guinea pigs, no response was observed either in the negative control group or in the test substance group in 24 hours and 48 hours after the patches were removed (Table 5). On the other hand, the positive control group was rated 3 in any observation times according to the standards by Magnusson and Kligman. Regarding sensitization rate, the test substance group was 0% and the positive group reached 100%. Thus, the test specimen did not sensitize skin. The intensity of sensitization of the test specimen was categorized as weak, which is the lowest category of I in the classification of Magnusson et al. We concluded that sugi CNF was negative in the skin sensitization

under the test condition.

4. Discussion and conclusion

There is a trend of asking safety verification for materials in everyday life, and in some cases safety verification of handling them in manufacturing. Even if the materials belong to products of natural origin, their scientific verification on safety is to be more demanded. We studied on the safety of sugi CNF prepared by enzymatic treatment followed by wet milling. The kinds of tests conducted were acute oral toxicity test using mice, bacterial reverse mutation test, chromosomal aberration test using Chinese hamster lung fibroblast cells, dermal irritation or corrosion tests using rabbits and skin sensitization test using guinea pigs. Sugi CNF was negative in any of those tests. In conclusion, sugi CNF was little possible to cause mutagenicity on organism or irritation on skin by contact under usual handling. As a bead mill was used for preparing the CNF, of which beads were made of zirconia, there might be a chance of contamination with fine zirconia particles. Bead mill that employs zirconia beads is commonly used

in milling food products as well as medical materials⁹⁾, and zirconia is supposed to be a durable element of high biocompatibility. It did not cause any trouble in our tests, and zirconia particles did not induce any doubt on its safety. Accumulating the verification results regarding the safety of CNF from various perspectives will be important to develop its demands in different kinds of industries.

Acknowledgement

The study was a part of the demonstrative project of cellulose nano-fiber manufacturing technology that was a part of the project of creating new demand for wood in the emergency measures to increase demand for wood products by Forestry Agency in 2014–2015. We are grateful to those concerned for their supports.

References

- 1) Magara K. : Seibutsushigen, 10 (3), 2–13 (2016)
- 2) Shimokawa T., *et al.* : Forestry and Forest Product Research Institute, Results of the 4th Midterm Plan 29 (2019). <https://www.ffpri.affrc.go.jp/pubs/chukiseika/4th-chuukiseika29.html>
- 3) Ishikawa A., *et al.* : Mokuzaishozon, 45 (2), 68–76 (2019)
- 4) Doak, S. H., *et al.* : Mutat. Res. 745, 104–111 (2012)
- 5) Ema M., *et al.* : Jpn. J. Environ. Toxicol., 14 (1), 81–97 (2011)
- 6) Shimokawa T., *et al.* : Bull. Forestry Forest Products Res. Inst., in press (2020)
- 7) Hayashi N., *et al.* : Forestry and Forest Product Research Institute, Results of the 3rd Midterm Plan 38–39 (2016)
- 8) Magnusson B., Kligman A. M. : J. Invest. Derm. 52 (3), 268–276 (1969)
- 9) Ban, S. : The Shikwa Gakuho, 107 (6), 670–684 (2007)