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Genotoxicity assessment of cellulose nanofibrils using a standard battery of *in vitro* and *in vivo* assays

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ABSTRACT

Cellulose nanofibrils (CNFs) are identified as novel nanomaterials with many potential applications. Since CNFs are fibrous manufactured nanomaterials, their potential carcinogenic effects and mesothelial toxicity raise some concerns. In this study, we conducted a standard battery of *in vitro* and *in vivo* assays to evaluate the genotoxicity of two CNF types using different manufacturing methods and physicochemical properties. Namely, one was CNF produced *via* chemical modification by TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical)-mediated oxidation, while the other was CNF produced *via* mechanical defibrillation using needle bleached kraft pulp. A bacterial reverse mutation test and a mouse lymphoma TK assay revealed that CNFs at 100 μg/mL did not induce bacterial reverse mutations and *in vitro* mammalian cell gene mutation. Further, *in vitro* chromosomal aberration tests demonstrated that CNFs at 100 μg/mL did not induce chromosomal aberration in Chinese hamster lung fibroblasts. From the mammalian erythrocyte micronucleus test, no statistically significant increase was observed in the proportion of micronucleated polychromatic erythrocytes in the bone marrow cells of rats intratracheally instilled with any concentration of CNFs (0.25–1.0 mg/kg) compared with values from respective negative control groups. Therefore, this battery of *in vitro* and *in vivo* assays illustrated that the CNFs examined in this study did not induce genotoxicity, suggesting our results provide valuable insight on the future use of these materials in various industrial applications.

1. Introduction

Cellulose nanofibrils (CNF) are identified as novel plant-derived nanomaterials that are low weight and high strength and have low thermal expansion coefficients, gas barrier properties, transparency, thickening properties, and thixotropic properties with broad, potential applications as alternatives to petroleum-derived materials [1–3]. Leveraging these characteristics has potential in various applications, such as automobiles, home electric appliances, electronic parts, packaging materials, filtration materials, inks, paints, cosmetics, and foods [4]. In 2019, the global outlook on cellulose nanomaterials showed an estimated turnover of approximately USD$700 million by the year 2024 [5].

In the future, CNF production, use, and disposal are expected to increase. Therefore, for CNFs to be widely used in society, human health and environmental hazard and exposure assessments are needed to confirm their safety. To ensure safety for humans (*e.g.*, workers and consumers) and the environment, further studies on CNF hazards, including inhaled, oral, and dermal toxicity, as well as genotoxicity and ecotoxicity, are needed. Exposure to synthetic carbon nanomaterials, including carbon nanofibers and carbon nanotubes (CNTs), has been considered a potential health hazard due to physical similarities with asbestos fibers [6]. However, knowledge of potential health effects is limited [7,8]. Accelerating the practical use of CNFs in society requires hazard assessments. Promoting safe commercialization is necessary to ensure safety, especially during workplace handling [9]. A better understanding of the risks associated with inhaling dried nanocellulose powder in the workplace is a high priority [10]. Considering exposure scenarios, CNF contact is expected to be inhaled or dermal, with the primary target organs being the lungs and skin. Several studies have revealed the pulmonary toxicity of CNFs [11–14]. However, recommendations for occupational exposure limits for CNF by inhalation or skin exposure are limited due to the scarcity of data available on its potential hazards.

Furthermore, considering the nanofibrous structure of CNFs, it is important to assess mutagenicity and genotoxicity potencies. While

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several genotoxicity assessments using single tests have been reported [15–18], using a standard battery of genotoxicity tests covering a wide range of mechanisms is essential to further clarify CNF genotoxicity. A standard battery of genotoxicity tests are defined by the following two options for testing pharmaceuticals based on the guidance of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use [19]. Option 1: (i) a test for gene mutation in bacteria; (ii) a cytogenetic test for identifying chromosomal damage, or an *in vitro* mouse lymphoma TK gene mutation assay; (iii) an *in vivo* test for genotoxicity, which is generally a test for detecting chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells. Option 2: (i) a test for gene mutation in bacteria; (ii) an *in vivo* assessment of genotoxicity with two different tissues, which is usually an assay for micronuclei using rodent hematopoietic cells and a second *in vivo* assay.

The safety evaluation methods proposed by the Organization for Economic Co-operation and Development (OECD) are commonly used internationally (http://www.oecd.org/env/ehs/testing/). In this present study, we assessed CNF genotoxicity using a standard battery of *in vitro* and *in vivo* assays, including an Ames test, an *in vitro* mammalian cell gene mutation test (mouse lymphoma TK assay: MLA assay), a chromosomal aberration test using mammalian cultured cells, and a micronucleus test. These studies were conducted following the OECD test guidelines. Further, the Ames test is not recognized as an informative component of a genotoxicity test for assessing nanomaterials [20] due to the Gram-negative strains of bacteria used in the test lacking the capacity for nanoparticle uptake, as well as mammalian mechanisms of endocytosis, pinocytosis, and phagocytosis. However, the mechanism of Gram-negative bacteria response to CNF exposure is yet to be determined. Therefore, in this study, we performed the Ames test as an *in vitro* mutagenicity assay.

CNFs have intermediate properties between gel and sol, and their viscosity is known to change with time and shear stress (*i.e.*, thixotropy). CNFs are often characterized as nanosized objects, with an aspect ratio of typically >10 and may exhibit longitudinal divisions, entanglements between particles, or network-like structures. The dimensions are typically 3–100 nm in diameter and up to 100 μm in length [21]. Meanwhile, the physicochemical properties of CNFs, such as fiber diameter, fiber length, morphology, functional groups, and impurities, can vary depending on raw materials used and chemical-mechanical treatment applied [22–24]. The safety of different grades of fibrillated celluloses should be assessed case by case [16]. For this study, we selected CNFs produced *via* (1) chemical modification of TEMPO-oxidized CNFs and successive mild disintegration in water and (2) mechanical defibrillation of needle bleached kraft pulp, as representative CNFs.

2. Materials and methods

2.1. Test materials and their preparation

Aqueous slurries of 10 mg/mL TEMPO-oxidized CNFs (hereinafter referred to as "CNF1″) and 20 mg/mL CNFs produced *via* mechanical defibrillation (referred to as "CNF2″) were obtained from Nippon Paper Industries Co. Ltd. (Tokyo, Japan) and Daio Paper Corporation (Tokyo, Japan), respectively. CNF1 suspensions containing organic nitrogen and sulfur compound preservatives were prepared using a planetary centrifugal bubble free mixer (ARE-310, THINKY CORPORATION, Tokyo, Japan) for 60 min. CNF2 containing 10 μg/mL benzalkonium chloride (BAC) was then prepared using an ultrasonic mixer (PR-1, THINKY CORPORATION, Tokyo, Japan) for 3 min [25]. Each aqueous suspension was adjusted to a concentration of 0.5–2.0 mg/mL for physicochemical and biological characterization, as well as genotoxicity tests.

2.2. Physicochemical and biological properties of CNFs in aqueous suspensions and cell culture medium

We used an H-7600 transmission electron microscopy (TEM) system at 80 kV (Hitachi, Ltd., Tokyo, Japan) to observe CNFs in the aqueous suspensions. Uranyl acetate was used for TEM staining. CNF diameters and lengths of the CNFs were measured from approximately 250 or 1000 CNFs using a JEM-1010 TEM (JEOL Ltd., Tokyo, Japan) at 100 kV. The rheological properties of aqueous CNF suspensions were measured using an MCR-302 rheometer (Anton Paar, Graz, Austria) over the shear rate range of 0.1–1000 s^{-1} (*D*), according to the manufacturer's procedure. The number of bacterial or fungal colonies and mycoplasma contamination were detected in two CNF aqueous suspensions and cell culture medium for *in vitro* genotoxicity assays using 3 M™ Petrifilm™ (3 M Japan Limited, Tokyo, Japan) and MycoAlert™ Mycoplasma Detection Kit (Lonza, Tokyo, Japan).

2.3. Bacterial reverse mutation test (Ames test)

Bacterial reverse mutation tests were conducted as per the OECD Guideline for Testing of Chemicals 471 "Bacterial Reverse Mutation Test" [26]. We cultured histidine-requiring *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and tryptophan-requiring *Escherichia coli* mutant WP2*uvrA* in nutrient broth at 37 °C with shaking. 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, NaN_3, 9-aminoacridine hydrochloride, and 2-aminoanthracene were used as positive controls [27]. The tests were performed after preincubation in the presence or absence of S9 mix (Oriental Yeast Co. Ltd., Tokyo, Japan). The vehicle was then used as the negative control. As a preliminary test, the appropriate concentration range for the main study was determined by observing an increase in the number of reverse mutation colonies compared with negative control groups in either the presence or absence of the S9 mix. All strains were tested at concentrations of 3.13–100 μg/plate CNFs using diluted 1.0 mg/mL aqueous suspension. The main test was performed at a CNF concentration based on preliminary tests results. For both main and preliminary tests, duplicate plates were used at each concentration. The results were considered positive if a twofold or larger increase was observed in the number of revertant colonies in the CNF-treated groups compared with the findings for the negative control, illustrating a concentration–response relationship.

2.4. In vitro mammalian cell gene mutation test (mouse lymphoma TK assay)

We conducted *in vitro* mammalian cell gene mutation tests (mouse lymphoma TK assay: MLA assay) as per the OECD Guideline for Testing of Chemicals 490 "*In Vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene," using 96-well microtiter plates [28]. The basic medium, designated as R-0, consisted of RPMI 1640 medium (Thermo Fisher Scientific K.K., Tokyo, Japan) supplemented with 200 μg/mL sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. Growth medium, designated as R-10, consisted of R-0 with 10 % (v/v) heat-inactivated horse serum. The cloning medium used for colony formation measurement, designated as R-20, consisted of basic medium with 20 % (v/v) heat-inactivated horse serum. The mouse lymphoma cell line L5178Y tk+/−3.7.2C was cultured in R-10 in a humidified incubator with 5% CO_2 at 37 °C. Methyl methanesulfonate (MMS; Sigma-Aldrich Japan, Tokyo) was used as the positive control for short-term exposure (3 h, in the absence of S9 mix) and continuous exposure (24 h, in the absence of S9 mix) experiments. Cyclophosphamide hydrate (CP; Shionogi & Co., Ltd., Osaka, Japan) was used as the positive control for short-term exposure experiments (3 h, in the presence of S9 mix). The test chemical solvent was used as the negative control. A preliminary test was performed to determine the cytotoxicity of CNFs at concentrations of 3.13–100 μg/mL using diluted 1.0 mg/mL aqueous suspension. The cytotoxicity was determined by the relative

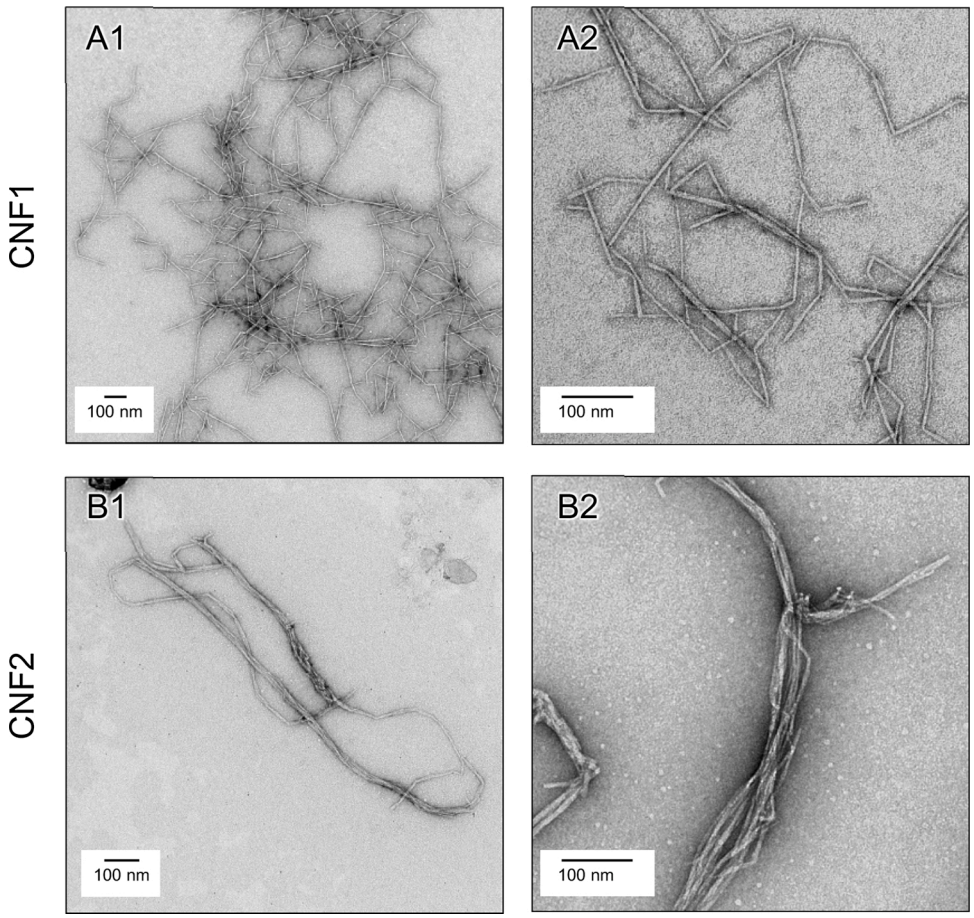

Fig. 1. TEM images of CNF1 and CNF2 suspensions. TEM, transmission electron microscopy; CNF, cellulose nanofibril.

suspension growth (RSG) and relative total growth (RTG). In the main study, the maximum concentration was set at 10 %–20 % RTG based on the OECD test guidelines [28]. Cells were plated at a density of 10^4 cells/mL in 96-well plates to evaluate cell cloning efficiency (CE) after incubation at 37 °C for 12 days. The ratio of CE in each treatment group to the negative control group was calculated as the relative cloning efficiency (RCE), after which RTG was calculated by multiplying RSG and RCE. Two more replicates per experimental group were exposed to 4 μg/mL trifluorothymidine (TFT) for mutation analysis. Plates were incubated at 37 °C for 12 days. The mutant colonies of each plate were counted using the naked eye. The colony size (small or large) was estimated in a similar manner to that described in the OECD Guideline for Testing of Chemicals 490 [28]. Induced mutant frequency (IMF) was calculated by subtracting the negative control (or untreated control) MF from the test culture MF. The global evaluation factor (GEF) was then defined as 126×10^{-6} [29].

2.5. *In vitro* mammalian chromosomal aberration test

In vitro mammalian chromosomal aberration tests were conducted as per the OECD Guideline for Testing of Chemicals 473 "*In vitro* Mammalian Chromosomal Aberration Test" [30]. We cultured the Chinese hamster lung fibroblast cell line CHL/IU in Eagle's minimum essential medium (Thermo Fisher Scientific K.K.) containing 10 % heat-inactivated bovine serum (Thermo Fisher Scientific K.K.). CHL/IU cells were incubated in a 5% CO_2 atmosphere at 37 °C, and the vehicle was used as the negative control. A preliminary cytotoxicity test was performed for cell growth inhibition at concentrations of 6.25–100 μg/mL CNFs using diluted 1.0 mg/mL aqueous suspension. We then performed the primary test at a CNF concentration determined from preliminary test results. Mitomycin C (Kyowa Kirin Co., Ltd., Tokyo, Japan) and CP (SHIONOGI) were used as positive controls, whereas duplicate plates were used at each concentration. The test was performed in the presence or absence of metabolic activation using S9 mix. The experiments included short-term exposure (6 h, in the presence or absence of S9 mix) and continuous exposure (24 h, in the absence of S9 mix). In both experiments, colcemid at a final concentration of 0.2 μg/mL (Thermo Fisher Scientific K.K.) was added 2 h before cell harvesting. Chromosomal preparations were then air-dried and stained with 1.2 % (v/v) Giemsa solution (Sigma-Aldrich) for 15 min at room temperature. Finally, we examined 150 metaphases/slide (300 metaphases/dose) for structural and numerical aberrations.

2.6. *Mammalian erythrocyte micronucleus test*

We conducted mammalian erythrocyte micronucleus tests as per the OECD Guideline for Testing of Chemicals 474 "Mammalian Erythrocyte Micronucleus Test" [31]. All animal tests were performed at the BioSafety Research Center Inc., Iwata, Japan. We purchased 8-week-old Crl:CD (Sprague–Dawley) rats from Charles River Laboratories Japan (Yokohama, Japan); these were then housed in metal cages in a room with 35 %–70 % humidity and a controlled temperature of 20 °C – 26 °C. Animals were fed a chow diet *ad libitum*. We performed a preliminary test for maximal tolerance in three male and three female rats. Because the lungs represent a major CNF exposure target, CNFs were intratracheally instilled once daily for 2 days at a dose of 0.5, 1.0, or 2.0 mg/kg body weight using 0.5, 1.0, or 2.0 mg/mL aqueous suspension. The preliminary test revealed no clear gender differences in toxicity between male and female rats (data not shown). Irregular respiration was observed in a few rats after intratracheal instillation at a dose of 2.0

Table 1
Characteristics of cellulose nanofibrils in the suspensions.

	Diameter Geometric mean (nm)	Geometric standard deviation	Length Geometric mean (μm)	Geometric standard deviation
CNF1	7.6	1.5	1.0	1.9
CNF2	21.2	2.0	1.7	2.0

mg/kg CNF1 and CNF2. Consequently, we used male rats instilled with CNFs at dosages of 0.25, 0.5, and 1.0 mg/kg body weight/day for the main study. CNFs were intratracheally instilled to six male rats per dose twice with an interval of 24 h. For the negative control, 1 mL/kg body weight of the vehicle was intratracheally instilled to six male rats for 2 days in a similar manner. For the untreated control group, we used five male rats without instillation. CP was intratracheally instilled in six male rats at a dose of 10 mg/kg body weight for 1 day as the positive control. We then excised femurs of each rat and flushed the bone marrow into test tubes using heat-inactivated bovine serum (Thermo Fisher Scientific K.K.). The percentage of micronucleated bone marrow polychromatic erythrocytes (MNPCEs) was calculated using 2000 polychromatic erythrocytes (PCEs) per rat. The percentage of PCEs among all erythrocytes was determined by counting 500 erythrocytes per rat. If a statistically significant difference in the frequency of micronucleated immature erythrocytes (MNIEs) was identified between the negative control and test substance groups, the result was then considered positive. However, final judgments were based on biological validity under test conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan, and the BioSafety Research Center Inc.

2.7. Statistical analysis

In mammalian erythrocyte micronucleus tests, we compared the frequency of MNIEs between the negative control and other groups using a conditional binomial test (Kastenbaum and Bowman's estimation method at a one-sided significance level of 0.025). When the frequency of MNIEs was significantly different between the negative control and test substance groups, the Cochran–Armitage trend test at a two-sided significance level of 0.05 was performed to confirm dose correlation. For the proportion of immature erythrocytes observed in red blood cells, we performed a Dunnett's multiple comparison at a two-sided significance level of 0.05 to compare negative control and test substance groups. We then used student *t*-tests at a two-sided significance level of 0.05 to compare untreated control and negative control groups and Welch's *t*-test at a two-sided significance level of 0.05 to compare untreated control and positive control groups.

3. Results

3.1. CNF characterization

TEM images of CNF1 suspensions showed a network structure of individually dispersed CNFs (Fig. 1). The geometric mean for CNF diameters and lengths were approximately 7.6 nm and 1.0 μm, respectively (Table 1). CNFs in the CNF2 suspension were comparatively dispersed with greater thickness and longer lengths, 21.2 nm and 1.7 μm, respectively. Partially entangled CNFs were observed in the CNF2 suspension. No noticeably aggregated CNFs were observed in all preparations (data not shown). CNF1 exhibited pseudoplastic flow behavior (Fig. 2, A1), and curves could be described using the pseudoplasticity equation, $\tau = kDn$. Apparent viscosity ($\eta = t/D$) suggested low shear rate dependence (Fig. 2, A2), and the CNF2 flow curve was observed to remain nearly constant Fig. 2, B1). Viscosity was almost inversely proportional to D (Fig. 2, B2). No bacterial or fungal colonies were detected in CNF1 or CNF2 cultured medium for *in vitro* genotoxicity assays (data not shown). The mycoplasma detection kit showed absence of mycoplasma contamination (negative result) in CNF1 or CNF2 aqueous suspension adjusted to a concentration of 1.0 mg/mL (data not shown).

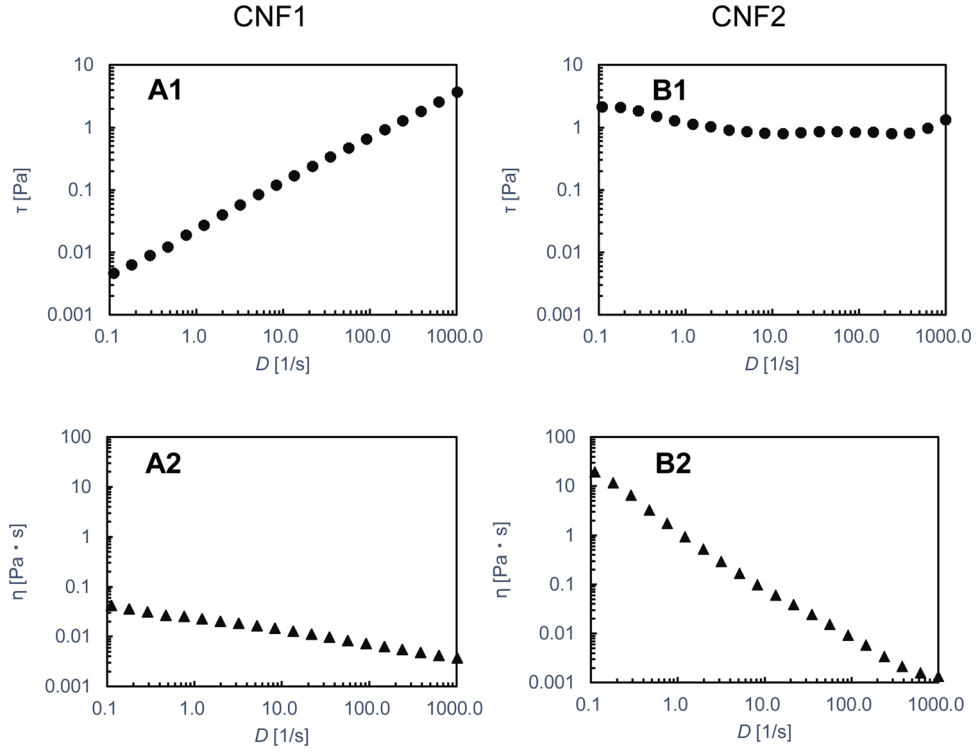

Fig. 2. Rheological properties of CNFs dispersed in CNF1 and CNF2 aqueous suspensions. Flow (A1 and B1) and viscosity curves (A2 and B2) for CNF1 and CNF2 are presented. CNF, cellulose nanofibril.

Table 2
Bacterial reverse mutation test in *S. typhimurium* or *E. coli* treated with cellulose nanofibrils.

Test substance	Concentration (μg/plate)	S9 mix	Number of revertant colonies per plate (Mean)				
			TA100	TA1535	WP2*uvrA*	TA98	TA1537
CNF1	0	–	97	11	20	20	8
	3.13	–	103	11	23	20	7
	6.25	–	122	12	30	26	4
	12.5	–	105	13	28	29	5
	25.0	–	101	12	30	19	8
	50.0	–	100	15	35	24	8
	100 †	–	124	11	24	30	7
AF-2	0.01	–	505		123		
	0.1	–				617	
NaN_3	0.5	–		555			
9-AA	80	–					216
CNF1	0	+	104	11	32	26	15
	3.13	+	104	13	24	20	8
	6.25	+	116	8	19	24	6
	12.5	+	119	12	19	34	9
	25.0 †	+	106	13	30	32	12
	50.0 †	+	136	10	21	30	15
	100 †	+	130	11	14	27	11
2AA	0.5	+				383	
	1	+	896				
	2	+		280			150
	10	+			617		
CNF2	0	–	96	8	25	24	7
	3.13	–	102	6	22	23	5
	6.25	–	95	11	22	21	8
	12.5 †	–	103	10	23	23	5
	25.0 †	–	96	7	21	26	5
	50.0 †	–	108	11	26	25	6
	100 †	–	111	10	31	26	7
AF-2	0.01	–	645		128		
	0.1	–				642	
NaN_3	0.5	–		585			
9-AA	80	–					242
CNF2	0	+	119	9	26	32	10
	3.13	+	114	9	23	36	12
	6.25	+	128	10	27	32	12
	12.5 †	+	124	10	24	22	10
	25.0 †	+	126	10	24	24	13
	50.0 †	+	109	11	22	33	10
	100 †	+	120	12	24	31	9
2AA	0.5	+				407	
	1	+	1097				
	2	+		295			189
	10	+			781		

Values are presented as the mean of two independent experiments.
AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide.
NaN_3: sodium azide.
9-AA: 9-Aminoacridine hydrochloride.
2-AA: 2-Aminoanthracene.
† Visible precipitation was observed by naked eye at the end of treatment period.

3.2. Ames test

Preliminary tests were conducted with six doses between 3.13 and 100 μg /plate. No twofold or greater increases were noted in the number of reverse mutation colonies compared with negative control groups for CNF1 and CNF2 suspensions in either the presence or absence of S9 mix (data not shown). The primary test was performed under the same dose conditions. Similarly, we observed no increase greater than twofold in the number of reverse mutation colonies for CNF1 and CNF2 suspensions in the presence or absence of S9 mix compared with negative control groups (Table 2). Conversely, the positive control substances indicated clear mutation-inducing effects on respective test strains.

3.3. Mouse lymphoma TK assay

From preliminary cytotoxicity tests, the RTGs in CNF1 suspensions at 3.13–100 μg/mL were 102.7 %, 87.4 %, 87.9 %, 81.1 %, 55.2 %, and 45.3 % for short-term (6 h) treatment in the absence of S9 mix; 105.0 %, 116.3 %, 117.1 %, 99.8 %, 75.0 %, and 38.5 % for short-term (6 h) treatment in the presence of S9 mix; and 102.3 %, 95.8 %, 123.4 %, 110.4 %, 73.8 %, and 62.3 % for continuous (24 h) treatment (Table 3). We observed precipitation at all treatment concentrations. The RTGs in the CNF2 suspensions at 3.13–100 μg/mL were 82.1 %, 78.2 %, 73.6 %, 64.4 %, 8.9 %, and 1.9 % for short-term treatment in the absence of S9 mix; 107.5 %, 90.0 %, 81.5 %, 59.2 %, 54.9 %, and 0.5 % for short-term treatment in the presence of S9 mix; and 92.7 %, 94.9 %, 92.7 %, 65.8 %, 23.1 %, and 4.7 % for continuous treatment in the absence of S9 mix. Precipitation was observed at all concentrations in short-term treatment in the absence of S9 mix, above 6.25 μg/mL in short-term treatment in the presence of S9 mix, and above 12.5 μg/mL in continuous treatment in the absence of S9 mix. In the main study, the IMF did not exceed a GEF value of 126×10^{-6} at any concentration of CNF1 or CNF2 for continuous treatment or short-term (6 h) treatment in the presence or absence of S9 mix (Table 4). IMF and CE of the negative control group were both within an acceptable range (IMF: 50–170 $\times$ 10^{-6}; CE: 0.65–1.20) [28]. Positive control MMS and CP showed high mutation frequency

Table 3
Growth inhibition for a mouse lymphoma TK assay in the mouse lymphoma cell line L5178Y tk+/−3.7.2C treated with cellulose nanofibrils.

Test substance	Concentration (μg/mL)	Short-term treatment (6 h), -S9 mix		Short-term treatment (6 h), +S9 mix		Continuous treatment (24 h), -S9 mix	
		RSG[a] (%)	RTG[b] (%)	RSG[a] (%)	RTG[b] (%)	RSG[a] (%)	RTG[b] (%)
CNF1	0	100.0	100.0	100.0	100.0	100.0	100.0
	3.13	85.6 †	102.7	110.7 †	105.0	102.3 †	102.3
	6.25	98.3 †	87.4	105.2 †	116.3	101.4 †	95.8
	12.5	83.0 †	87.9	92.7 †	117.1	106.8 †	123.4
	25.0	86.1 †	81.1	99.1 †	99.8	87.3 †	110.4
	50.0	55.3 †	55.2	76.7 †	75.0	73.8 †	73.8
	100	44.0 †	45.3	37.0 †	38.5	62.3 †	62.3
CNF2	0	100.0	100.0	100.0	100.0	100.0	100.0
	3.13	95.5 †	82.1	90.5	107.5	88.5	92.7
	6.25	83.6 †	78.2	87.6 †	90.0	107.9	94.9
	12.5	78.6 †	73.6	105.4 †	81.5	99.5 †	92.7
	25.0	66.9 †	64.4	70.4 †	59.2	72.7 †	65.8
	50.0	7.1 †	8.9	38.4 †	54.9	22.7 †	23.1
	100	1.7 †	1.9	0.3 †	0.5	4.6 †	4.7

[a] Relative suspension growth.
[b] Relative total growth.
† Visible precipitation was observed by naked eye at the end of treatment period.

compared with negative control groups for all treatments.

3.4. *In vitro* mammalian chromosomal aberration test

Preliminary cytotoxicity tests for the *in vitro* mammalian chromosomal aberration assays revealed no growth inhibition at CNF1 and CNF2 concentrations of 3.13, 6.25, 12.5, 25, 50, and 100 μg/mL following short-term (6 h) exposure in the presence or absence of S9 mix or continuous (24 h) exposure in the absence of the S9 mix (Table 5). Based on preliminary test results, the main test was conducted at CNF concentrations of 25, 50, and 100 μg/mL. CNFs did not increase the number of structural and numerical chromosomal aberrations for any concentration following short-term exposure in the presence or absence of the S9 mix or continuous exposure in the absence of S9 mix (Table 6). No statistically significant increase in the frequency of abnormal chromosomal and polyploid cells was observed in comparison with the negative control group. Mitomycin C and CP exposure markedly increased structural chromosomal aberrations.

3.5. *Mammalian erythrocyte micronucleus test*

For the micronucleus test, no statistically significant increase was observed in the proportion of MNPCEs in bone marrow in rats intratracheally instilled with any CNF concentration compared with the negative control group (Table 7). Furthermore, we observed no statistically significant reduction in the PCE ratio. The MNPCE proportion in the negative control group was within the standard value derived from background data, whereas the frequency of MNPCEs in the positive control group was significantly higher than the negative control group.

4. Discussion

In this present study, we have assessed two types of CNFs with different physicochemical properties for their potential genotoxicity using Ames tests, mouse lymphoma TK assays, *in vitro* mammalian chromosomal aberration tests, and mammalian erythrocyte micronucleus tests. In conclusion, a standard battery of *in vitro* and *in vivo* genotoxicity assay demonstrated no genotoxicity in the two CNFs in this study.

Several genotoxicity assessments have previously been performed by a single test, resulting in varying test materials and results. *In vitro* micronucleus assay data illustrated that CNF produced by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO)-mediated oxidation of an industrial bleached *Eucalyptus globulus* were genotoxic at low, but not high, concentrations [15]. A bacterial reverse mutation test (Ames test) using *Salmonella typhimurium* showed no genotoxic effect of fractionated fibrillated cellulose [16]. TEMPO-oxidized CNF administered to mice *via* pharyngeal aspiration induced an acute inflammatory response and DNA damage in the lungs, but no systemic genotoxic effects were observed in the bone marrow [17]. Further, ruby cotton nanofibers did not cause any significant DNA breaks [18]. However, to the best of our knowledge, no studies employed a standard battery of genotoxicity tests for CNFs.

Generally, a single genotoxicity test implemented using individual endpoints cannot determine all genotoxicity aspects, so several combined genotoxicity tests are recommended in order to clarify the genotoxicity of chemical substances. A standard battery testing approach is viable since no single test is capable of detecting all genotoxic mechanisms relevant in tumorigenesis. Genotoxicity assessment of novel chemical substances, such as pharmaceuticals using standard battery testing, has been identified to be essential in the field of regulatory toxicology [19]. Further, appropriate methodologies, including both *in vitro* and *in vivo* tests, are needed to investigate the genotoxic effects of nanoparticles [32]. Here, a standard battery of genotoxicity tests for manufactured nanomaterials is a practical, pragmatic approach [33]. Indeed, standard battery testing has also assessed the genotoxicity of manufactured nanomaterials [34–37]. Therefore, we believe that our findings using a standard battery of genotoxicity tests are useful for assessing CNF genotoxicity.

In this study, a standard battery of *in vitro* and *in vivo* genotoxicity assays showed no genotoxicity in our CNFs. However, this does not provide evidence that all CNFs lack genotoxicity. The two types of CNFs in this study were examples of chemically modified CNFs and CNFs produced *via* mechanical defibrillation of needle bleached kraft pulp. CNF manufacturing methods and types vary depending on application [5], highlighting the difficulty in reaching definitive conclusions regarding CNF genotoxicity. A weight of evidence analysis demonstrated differences in CNT genotoxicity, but further research is required to unravel the physicochemical characteristics that determine genotoxic risk [38]. A standard battery of *in vitro* and *in vivo* genotoxicity assays should thus be conducted simultaneously when investigating the physicochemical properties of each CNF.

This present study suggests two types of CNFs do not directly interact with DNA or cause mutations and clastogenic events. Meanwhile, indirect genotoxicity mechanisms can be defined as interactions with non-DNA targets leading to genotoxic effects [39]. Further research is needed to elucidate the mechanisms potentially involved in CNF-induced genotoxicity.

Due to the high aspect ratio of many CNFs, as well as CNTs, a fiber pathogenicity paradigm is a promising, future option. This fiber

Table 4
A mouse lymphoma TK assay in the mouse lymphoma cell line L5178Y tk+/−3.7.2C treated with cellulose nanofibrils.

Test substance	CNF Concentration (μg/mL)	Exposure time (h)	S9 mix	RSG[a] (%)	RTG[b] (%)	CE[c]	Mutation frequency ($x10^{-6}$)	L-MF[d] ($x10^{-6}$)	S-MF[e] ($x10^{-6}$)	IMF[f]
Non-treatment	–	6	–	100.7	100.6	0.893	91.7	45.5	42.4	–
CNF1	0	6	–	100.0	100.0	0.894	95.1	50.3	40.8	–
	6.25 †	6	–	100.7	79.6	0.706	160.8	73.8	77.9	65.7
	12.5 †	6	–	99.4	96.3	0.866	123.6	47.0	70.3	28.5
	25.0 †	6	–	95.7	84.9	0.793	80.5	33.7	44.2	−14.6
	50.0 †	6	–	72.2	70.0	0.866	80.5	30.9	47.0	−14.6
	100 †	6	–	42.3	30.7	0.649	145.3	58.3	80.3	50.2
MMS (10 μg/mL)	–	6	–	111.0	90.2	0.727	435.0	108.4	268.2	339.9
Non-treatment	–	6	+	126.5	104.2	0.748	109.5	54.4	50.6	–
CNF1	0	6	+	100.0	100.0	0.908	102.2	48.0	50.4	–
	6.25 †	6	+	114.0	105.6	0.841	75.9	41.7	35.1	−26.3
	12.5 †	6	+	92.8	85.9	0.841	93.7	41.7	51.7	−8.5
	25.0 †	6	+	100.8	108.8	0.980	102.7	56.1	41.5	0.5
	50.0 †	6	+	37.1	40.0	0.980	105.9	56.1	44.4	3.7
	100 †	6	+	41.4	36.1	0.793	111.0	47.7	58.5	8.8
CP (3 μg/mL)	–	6	+	46.7	30.6	0.596	1004.2	346.7	401.3	902.0
Non-treatment	–	24	–	174.2	196.0	0.866	112.5	63.5	43.7	–
CNF1	0	24	–	100.0	100.0	0.770	78.1	28.6	47.4	–
	6.25 †	24	–	100.4	127.8	0.980	86.7	24.5	59.1	8.6
	12.5 †	24	–	99.1	108.2	0.841	86.5	35.1	48.4	8.4
	25.0 †	24	–	79.4	81.8	0.793	122.9	44.2	73.0	44.8
	50.0 †	24	–	68.9	65.0	0.727	104.2	44.4	55.9	26.1
	100 †	24	–	43.6	50.6	0.893	81.5	26.9	51.9	3.4
MMS (5 μg/mL)	–	24	–	110.1	92.8	0.649	991.7	205.7	558.5	913.6
Non-treatment	–	6	–	114.1	140.3	1.160	97.9	30.2	62.7	–
CNF2	0	6	–	100.0	100.0	0.943	142.5	64.5	70.2	–
	0.410	6	–	87.4	80.2	0.866	162.1	63.5	87.5	19.6
	0.819	6	–	99.6	86.2	0.816	163.6	85.5	67.4	21.1
	1.64 †	6	–	88.1	94.5	1.012	142.1	63.0	68.9	−0.4
	3.28 †	6	–	99.9	97.6	0.921	167.6	66.1	92.2	25.1
	6.55 †	6	–	114.8	108.7	0.893	119.9	48.7	71.4	−22.6
	13.1 †	6	–	96.7	79.0	0.770	169.0	79.0	79.0	26.5
	26.2 †	6	–	80.2	71.5	0.841	162.8	62.0	93.7	20.3
	32.8 †	6	–	69.7	64.0	0.866	150.3	56.8	84.0	7.8
	41.0 †	6	–	33.5	19.4	0.547	207.5	95.3	100.5	65.0
	51.2 †	6	–	4.3	3.4	0.748	187.7	77.4	101.3	45.2
MMS (10 μg/mL)	–	6	–	101.3	112.3	1.046	383.9	105.4	232.7	241.4
Non-treatment	–	6	+	118.0	116.7	0.793	155.6	73.0	76.7	–
CNF2	0	6	+	100.0	100.0	0.802	163.3	75.9	79.7	–
	0.819	6	+	89.7	117.0	1.046	134.2	52.6	72.4	−29.1
	1.64	6	+	86.6	90.8	0.841	158.8	45.0	108.4	−4.5
	3.28	6	+	87.8	89.3	0.816	180.5	63.9	107.9	17.2
	6.55 †	6	+	104.8	92.2	0.706	194.0	86.2	98.8	30.7
	13.1 †	6	+	128.0	116.0	0.727	197.9	79.7	104.2	34.6
	26.2 †	6	+	114.5	106.8	0.748	187.7	85.3	89.3	24.4
	32.8 †	6	+	98.2	97.1	0.793	194.7	62.1	122.9	31.4
	41.0 †	6	+	81.2	99.2	0.980	143.2	59.1	77.3	−20.1
	51.2 †	6	+	69.5	63.0	0.727	188.4	87.8	91.8	25.1
	64.0 †	6	+	61.3	58.8	0.770	160.3	71.4	79.0	−3.0
	80.0 †	6	+	50.2	46.8	0.748	183.1	65.8	105.4	19.8
	100.0 †	6	+	38.3	36.8	0.770	200.5	71.4	118.4	37.2
CP (3 μg/mL)	–	6	+	43.4	21.4	0.395	1754.8	215.1	1140.2	1591.5
Non-treatment	–	24	–	206.2	165.8	0.667	104.6	48.4	56.8	–
CNF2	0	24	–	100.0	100.0	0.830	111.9	33.3	76.0	–
	1.64	24	–	112.9	111.0	0.816	123.3	16.2	104.1	11.4
	3.28	24	–	96.9	104.3	0.893	165.0	20.8	138.2	53.1
	6.55	24	–	100.9	105.2	0.866	91.0	24.6	63.5	−20.9
	13.1 †	24	–	102.6	110.4	0.893	105.6	23.8	78.1	−6.3
	26.2 †	24	–	72.3	94.3	1.082	96.0	24.7	67.2	−15.9
	32.8 †	24	–	63.9	70.9	0.921	126.8	26.1	95.6	14.9
	41.0 †	24	–	23.7	28.0	0.980	89.8	24.5	65.1	−22.1
	51.2 †	24	–	17.9	18.7	0.866	101.7	21.4	77.1	−10.2
	64.0 †	24	–	15.6	12.9	0.687	105.9	23.1	80.1	−6.0
	80.0 †	24	–	8.2	9.7	0.980	93.0	18.9	71.2	−18.9
MMS (5 μg/mL)	–	24	–	124.1	86.6	0.579	1161.9	60.5	976.1	1050.0

MMS, Methyl methanesulfonate; CP, cyclophosphamide.
[a] Relative suspension growth.
[b] Relative total growth.
[c] Cloning efficiency.
[d] Large colony mutant frequencies.

[e] Small colony mutant frequencies.
[f] Induced mutant frequency.
[†] Visible precipitation was observed by naked eye at the end of treatment period.

Table 5
Growth inhibition for chromosomal aberration test in Chinese hamster lung cell line treated with cellulose nanofibrils.

Test substance	Concentration (μg/mL)	Relative cell growth (%)		
		Short-term treatment (6 h), -S9 mix	Short-term treatment (6 h), +S9 mix	Continuous treatment (24 h), -S9 mix
CNF1	0	100.0	100.0	100.0
	3.13	88.9	101.6	118.8
	6.25	99.3	80.7	95.8
	12.5	96.5	109.4	88.8
	25.0	95.1	80.2	100.3
	50.0	93.1	101.6	110.5
	100	92.4	72.4	69.0
CNF2	0	100.0	100.0	100.0
	3.13	101.4 [†]	101.5	104.3
	6.25	111.6 [†]	100.9	102.9
	12.5	109.4 [†]	94.9	107.9
	25.0	83.3 [†]	100.9	92.1
	50.0	94.2 [†]	105.1	108.6
	100	99.3 [†]	92.4	111.4

[†] Visible precipitation was observed by naked eye at the end of treatment period.

paradigm has a robust structure/toxicity relationship to predict fiber pathogenicity depending on their length, thickness, and biopersistence [40]. Ede et al. conclude from a systematic review that neither CNF nor cellulose nanocrystal (CNC) appear to conform to the fiber paradigm [41]. However, little is known about CNF biopersistence. We have recently reported that the pulmonary inflammation caused by CNFs is mild compared with that caused by MWCNTs; however, CNFs deposited in alveolar macrophages were observed in rats at 90 days following intratracheal instillation, as well as MWCNTs [14]. Quantitative analysis of residual CNFs in the lung is extremely difficult due to bio-derived substances. However, based on our histopathological observations, CNF is not easily cleared from the lung following inhalation. Persistent deposition of poorly soluble substances in the lungs suggests potential for lung cancer development.

The International Agency for Research on Cancer designated certain Mitsui-7 multi-walled carbon nanotube (MWCNT-7) as a Group 2B carcinogen or "possibly carcinogenic to humans" [42]. Hence, the Japanese government distinguished MWCNT-7 from other CNTs and identified them as target substances under carcinogenicity guidelines. CNFs, like CNTs, need to be examined for their carcinogenic effects, particularly in working environments. There is a current knowledge gap in identifying carcinogenic mechanisms, which will be a subject for future research. We believe evaluating genotoxicity by a standard battery of *in vitro* and *in vivo* genotoxicity assays, as per this study, will serve as convincing evidence to continue predicting potential CNF carcinogenicity.

Table 6
Chromosomal aberration test in Chinese hamster lung cell line treated with cellulose nanofibrils.

Test substance	Concentration (μg/mL)	Exposure time (h)	S9 mix	No. of cells	Structural (%)		Polyploid cells (%)	Relative cell growth (%)
					+ Gap	- Gap		
CNF1	0	6	–	300	1.7	1.3	0.0	100.0
	25.0	6	–	300	1.3	1.0	0.0	93.4
	50.0	6	–	300	1.3	0.7	0.3	93.0
	100	6	–	300	1.0	1.0	0.0	95.8
MMC	0.1	6	–	300	71.3 *	69.3 *	0.0	73.0
CNF1	0	6	+	300	0.3	0.3	0.7	100.0
	25.0 [†]	6	+	300	1.0	0.7	0.3	92.6
	50.0 [†]	6	+	300	0.3	0.3	0.7	96.8
	100 [†]	6	+	300	0.7	0.7	0.3	97.4
CP	12.5	6	+	300	53.0 *	52.3 *	0.0	71.9
CNF1	0	24	–	300	1.3	1.0	0.0	100.0
	25.0	24	–	300	2.7	2.0	0.0	102.6
	50.0	24	–	300	2.3	2.0	0.7	101.1
	100	24	–	300	2.0	2.0	0.0	104.3
MMC	0.05	24	–	300	50.0 *	49.0 *	0.0	57.3
CNF2	0	6	–	300	1.0	0.7	0.3	100.0
	25.0 [†]	6	–	300	1.3	1.0	0.3	104.6
	50.0 [†]	6	–	300	1.3	0.7	0.3	82.8
	100 [†]	6	–	300	1.0	1.0	0.0	97.3
MMC	0.1	6	–	300	38.0 *	37.0 *	0.0	89.7
CNF2	0	6	+	300	0.0	0.0	0.0	100.0
	25.0 [†]	6	+	300	0.0	0.0	0.0	90.1
	50.0 [†]	6	+	300	0.0	0.0	0.3	100.3
	100 [†]	6	+	300	1.3	1.3	0.3	92.9
CP	12.5	6	+	300	59.0 *	58.0 *	0.0	70.8
CNF2	0	24	–	300	0.7	0.7	0.3	100.0
	25.0 [†]	24	–	300	1.3	1.3	0.0	121.0
	50.0 [†]	24	–	300	0.7	0.7	1.0	126.6
	100 [†]	24	–	300	1.0	0.7	1.3	125.9
MMC	0.05	24	–	300	29.7 *	29.0 *	0.0	98.6

- Gap: total number of cells with aberrations except gap.
MMC, mitomycin C; CP, cyclophosphamide.
NA: Not analyzed.
[†] Visible precipitation was observed by naked eye at the end of treatment period.
[*] Significant difference from negative control (Fisher's exact test): $p < 0.025$.

Table 7
Micronucleated polychromatic erythrocytes observed in bone marrow cells of male rat intratracheally instilled with cellulose nanofibrils.

Test substance	Dose (mg/kg)	Number of animals	% MNPCE (Mean ± SD)[a]	% PCE (Mean ± SD)[b]
Untreated	–	5	0.17 ± 0.03	52.9 ± 4.0
CNF1	0	5	0.13 ± 0.08	54.6 ± 3.1
	0.25	5	0.13 ± 0.06	50.5 ± 4.8
	0.5	5	0.13 ± 0.04	53.5 ± 7.9
	1.0	5	0.09 ± 0.07	54.7 ± 3.5
CNF2	0	5	0.17 ± 0.09	53.6 ± 9.6
	0.25	5	0.15 ± 0.09	57.8 ± 4.9
	0.5	5	0.16 ± 0.11	57.0 ± 3.5
	1.0	5	0.14 ± 0.08	55.1 ± 5.1
CP	10	5	2.08 ± 0.28 *,‡	51.6 ± 3.3

MNPCE: Micronucleated polychromatic erythrocyte.
PCE: Polychromatic erythrocyte.
CP: Cyclophosphamide, p.o., 10 mL/kg.
[a] Two thousand PCE were analyzed per animal, for a total of 10,000 cells per group.
[b] Five hundred normochromatic erythrocytes were analyzed, for a total of 2500 cells per group.
* Significant difference from negative control ($p < 0.025$).
‡ Kastenbaum and Bowman (KB) method.

5. Conclusion

In this present study, TEMPO-oxidized CNFs and CNFs produced *via* mechanical defibrillation were assessed for their potential genotoxicity using a standard battery of *in vitro* and *in vivo* genotoxicity assays. Ames tests highlighted two CNF types in aqueous suspensions did not induce genetic mutations in *S. typhimurium* or *E. coli*. An MLA assay demonstrated that CNFs did not induce *in vitro* mammalian cell gene mutation. Further, CNFs did not increase the number of structural chromosomal aberrations or numerical chromosomal aberrations, regardless of metabolic activation. In the micronucleus test, no statistically significant increase was noted in the proportion of MNPCEs in rat bone marrow intratracheally instilled with any CNF concentration compared with negative control groups. Furthermore, we observed no statistically significant reduction in PCE ratio. The standard battery of *in vitro* and *in vivo* genotoxicity assays demonstrated that CNFs exhibited no genotoxicity, confirming the CNFs used in this study were non-genotoxic and served as data to dispel concerns about CNF production from manufacturing and processing in working environments.

CRediT authorship contribution statement

Katsuhide Fujita: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing, Project administration. **Sawae Obara:** Data curation, Validation, Writing - review & editing, Visualization. **Junko Maru:** Data curation, Validation, Visualization. **Shigehisa Endoh:** Data curation, Visualization.

Declaration of Competing Interest

The authors declare no conflict of interest.

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