Physical, chemical, and toxicological characterization of sulfated cellulose nanocrystals for food-related applications using in vivo and in vitro strategies

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Abstract

Cellulose nanocrystals (CNCs) are a next-generation cellulose product with many unique properties including applications in the food industry as a food additive, food coating, and in food-contact packaging material. While CNC is anticipated to be safe due to its similarity to the many forms of cellulose currently used as food additives, special consideration is given to it as it is the first manufactured form of cellulose that is nanoscale in both length and width. A proactive approach to safety has been adopted by manufacturers to demonstrate CNC safety toward responsible commercialization. As part of the safety demonstration, in vivo and in vitro testing strategies were commissioned side-by-side with conventional cellulose, which has been safely used in food for decades. Testing included a 90-day rodent feeding study as well as additional physical, chemical, and biological studies in vitro that follow European Food Safety Authority (EFSA) guidance to demonstrate the safe use of novel food ingredients. The strategy includes assessment of neat materials side-by-side with simulated digestion, mimicking conditions that occur along the gastrointestinal tract as well as intracellularly. An intestinal co-culture model examined any potential toxicological effects from exposure to either pristine or digested forms of CNC including cytotoxicity, metabolic activity, membrane permeability, oxidative stress, and proinflammatory responses. None of the studies demonstrated any toxicity via oral or simulated oral exposure. These studies demonstrate that CNC produced by InnoTech Alberta is similarly safe by ingestion as conventional cellulose with a no-observed-adverse-effect level of 2085.3 (males) and 2682.8 (females) mg/kg/day.

Key words: cellulose nanocrystals; 90-day subchronic study; NOAEL; intestinal model; in vitro toxicology; simulated digestion; alternative testing strategy

Introduction

Cellulose is the fundamental structural component of plants and is also naturally produced by invertebrates, algae, bacteria, and fungi, making it the most abundant natural polymer on earth [1]. Mechanical and chemical refinement of wood pulp is used to isolate purified cellulose fibers and produce a variety of different morphological and functional cellulose derivatives.
that are widely used in commerce [2]. Several forms of cellulose have been used as food-additives and food-contact materials for several decades and are designated as Generally Recognized As Safe (GRAS) by the US Food and Drug Administration [3]. As a food additive, cellulose and its derivatives have been used for the unique technical effects they impart to food including as a rheology modifier, stabilizer, low-calorie substitute, fiber supplement, and processing aid [4].

Sulfated cellulose nanocrystal (CNC) is a next-generation cellulose product manufactured by further chemical and mechanical processing of cellulose fibers to release and isolate the crystalline portion of individual cellulose fibrils, a major component of cellulose’s structural building block [1]. CNC has many unique properties such as high tensile strength, enabling high capacity thermal and mechanical applications in paper, textiles, pharmaceuticals, filtration devices, insulation, building materials, and more [1]. CNC is useful in the food industry as a food additive, food coating, and in food-contact packaging materials [5]. In particular, the barrier properties of these bio-based materials provide promising alternatives to conventional petroleum materials such as plastic packaging [6].

CNC is anticipated to be as safe as the many related forms of cellulose currently used as food additives; however, given this is the first manufactured form of cellulose that is nanoscale in both length and width, a proactive approach to safety has been adopted by several manufacturers to demonstrate its safety and promote responsible commercialization [7]. A food safety study was commissioned by a consortium of 10 organizations partnering with academic, governmental, and commercial labs to assess the safe use of two novel forms of cellulose in food: fibrillated cellulose and nanocrystalline cellulose. The testing strategy adopted a tiered approach to complete a full physical, chemical, and biological characterization of these novel forms of cellulose side-by-side to a conventional cellulose (CC) material, Solka Flocc®, using both traditional and next-generation testing. Results for fibrillated forms of cellulose that demonstrated these materials are as safe as traditional forms of cellulose and are safe to use in food up to 4% by weight [7, 8]. Here, we present the results for the nanocrystalline form according to this testing strategy, demonstrating that it is similarly safe for use in food-related applications.

As part of the safety demonstration for CNC, the consortium commissioned a 90-day rodent feeding study following Organization for Economic Cooperation and Development (OECD) Test Guideline (TG) 408. Such studies are most commonly requested by regulators in markets worldwide as part of a safety demonstration for food additives. In addition, the consortium commissioned the design of additional physical, chemical, and biological studies in vitro to demonstrate the similarity of CNC to conventional forms of cellulose. A number of international organizations and governments have called for the drastic reduction of animal testing through the use of alternative test methods that reduce, refine, or replace vertebrate testing [9], while some governments have implemented related regulations [10]. Performing side-by-side in vivo and in vitro testing supports this goal and helps refine and validate alternative test methods, especially toward the next generation of these novel materials.

The in vitro strategy adheres to EFSA guidance toward demonstrating the safe use of novel food ingredients and is intended to limit the need for excessive animal testing to demonstrate the safety of chemically similar materials [11]. The in vitro testing includes simulated digestion of CNC mimicking conditions that occur along the gastrointestinal tract (mouth, stomach, and intestine) as well as intracellularly (lysosomes). A comprehensive physical and chemical characterization of CNC in its pristine form, as well as post-digestion, side-by-side with CC, compares these materials and examines whether any changes in structure may be anticipated as the material undergoes digestion. Finally, a fully characterized intestinal co-culture model mimicking the physiology of the human intestine is used to examine any potential toxicological effects from exposure to either pristine or digested forms of CNC. Endpoints examined include cytotoxicity, metabolic activity, membrane permeability, oxidative stress, and proinflammatory responses. Results from the tiered in vivo and in vitro testing strategy for CNC are presented here, demonstrating that it is as safe as CC to use as a food additive ingredient up to 4% of the diet.

Materials and Methods

Experimental overview

Figure 1 describes this study’s experimental design. Cellulose materials (CNC and CC) underwent standardized sample dispersion, simulated gastrointestinal digestion (at 0.25, 0.5, 1, and 4 h), and simulated lysosomal digestion (at 0.5, 2, 24, and 72 h). Samples suspended following a standardized sample dispersion were labeled “pristine materials”; samples digested through simulated gastrointestinal fluid were labeled “digested materials”; and samples incubated in simulated lysosomal fluid were labeled “lysosomal digested materials.” All 10 samples of pristine and digested CNC and CC and the 8 lysosomal digested CNC and CC were physically and chemically characterized including Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS), and inductively coupled plasma mass spectrometry (ICP-MS). The pristine and digested cellulose samples (CNC and CC) were then biologically tested in an in vitro gastrointestinal co-culture model over time (1, 6, 24, and 48 h) and measured for metabolic activity, proinflammatory effects, oxidative stress, cell viability, and barrier integrity over 7 days of culture (transepithelial electrical resistance; TEER).

Materials

InnoTech Alberta, in collaboration with Alberta-Pacific Forest Industries Inc. (Al-Pac) (Edmonton, Alberta, Canada) supplied CNC (CAS Registry Number 9005-22-5). CNC is produced through sulfuric acid hydrolysis of wood pulp, purification, and subsequent neutralization with sodium hydroxide. CNC was provided as a spray-dried powder (100% wt.) For details of CNC production, see Ngo et al. [12].

CC (Food-grade Solka Floc, grade FCC200, CAS number 9004-34-6) was purchased from Solvaira Specialty LP. Solka Floc is GRAS in the USA and sold commercially as a fiber ingredient for use as a bulking agent, thickener, stabilizer, anti-caking agent, texturizing agent, and more. It served as a CC control group to compare with CNC.

CNC was assessed for impurities following the methods and specifications outlined the Food Chemicals Codex (FCC) 11th edition, including chloride, lead, sulfur, ash, loss on drying, pH, and water-soluble substances (Food Chemicals Codex 2016). Solvaira Specialty LP provided a Certificate of Analysis for CC. All parameters were within FCC specifications for CNC and CC. There was no microbiological contamination, as determined by standard plate count; yeast and mold contamination by plate count method; and salmonella, according to methods and specifications recommended by the FCC (2016).
In vivo testing

The study was conducted according to current OECD Test Guideline 408 [13] and US FDA guidance (FDA 2007), under standards of GLP [14] at Product Safety Labs (Dayton, NJ), a member of the Association for Assessment and Accreditation of Laboratory Animal Care following National Research Council (2011) guideline standards.

Feed preparation. Open Standard Diet D1112219N Rodent Diet and addition of celluloses to feed was performed by Research Diets Inc. (New Brunswick, NJ). Control and test diets were formulated by adding CC or CNC to the standard basal diet to achieve the target doses of 2, 3, and 4 w/w% cellulose. Comparable fat, protein, and carbohydrate contents in the diet were maintained across dose groups and between CC and CNC groups.

Study overview. One hundred and twenty male and female Sprague Dawley CD® IGS rats (6–7 weeks; Charles River Laboratories, Raleigh, NC) were acclimated in suspended stainless steel cages for 5 days then randomly assigned to control or treatment groups. Each group had 10 female and 10 male rats. Rats were maintained on a 12-h light/dark cycle, with a temperature ranging from 19 to 23 °C. Body weights and clinical observations were recorded twice prior to study start. Target doses of 2, 3, and 4% CNC in the diet were selected on the basis of two range finding studies (based on OECD TG 407), which determined that there were no adverse effects associated with feeding 5% CNC over 7 days or with feeding up to 1.2% CNC over 14 days. Doses were based on anticipated use-levels of CNC as a food additive.

The study included three control groups fed diets with 2, 3, and 4% CC so that any adverse effects resulting from higher or lower dietary fiber intake could be distinguished from effects related to the CNC test material itself (in comparison to CC at the same concentration). The diets were provided ad libitum, except for a fasting period prior to blood collection.

Study endpoints. Clinical observations. All animals were observed at least twice daily for mortality, and cage-side observations were recorded daily. Weekly detailed clinical observation was conducted while handling the animal. Observations included abnormal morphology or activity, including visible changes to skin, fur, eyes, or mucous membranes; secretions and excretions; autonomic activity (e.g. lacrimation, piloerection, pupil size, and unusual respiratory patterns); changes in gait, locomotion (speed and vigor of movement), posture, and response to handling, as well as the presence of clonic or tonic movements, stereotypes (e.g. excessive grooming and repetitive circling), vocalizations, and bizarre behavior (e.g. self-mutilation and walking backward).

Body weight, food consumption, body weight gain, organ weight. Individual body weights were recorded twice during acclimation, then weekly. Individual food consumption was recorded at the same time as body weight measurements. Animals were housed individually in suspended steel cages; any uneaten food fell through the bottom of the cage to a drop pan and was weighed for accurate calculation of food consumption. The adrenals, kidneys, testes, brain, liver, thymus, epididymides, ovaries with oviducts, uterus, heart, and spleen were weighed wet after sacrifice, and relative organ weights (organ weight to total body weight) were calculated.

Ophthalmology. During the acclimation period and prior to test termination, the eyes of rats were examined by focal illumination, indirect ophthalmoscopy, and, if necessary, slit-lamp microscopy.

Clinical pathology. Clinical pathology evaluations included assessment of hematology, serum chemistry, urinalysis, and post-mortem anatomic pathology. One day prior to sample collection, the animals were fasted overnight. Blood samples were collected via sublingual bleeding under isoflurane anesthesia during Week 12. For hematology assessments, ~500 μL of blood was collected in pre-calibrated tubes with K2-ethylenediaminetetraacetic acid. For clinical chemistry assessments, ~1000 μL of blood was collected into a tube containing no preservative. Blood samples used to determine the prothrombin time and activated partial thromboplastin time (coagulation) were collected via the inferior vena cava under isoflurane anesthesia at terminal sacrifice. Approximately 1.8 mL of blood was collected in pre-calibrated tubes with 3.2% sodium citrate. All blood samples were prepared for storage in a refrigerated centrifuge and placed in a −80°C freezer. For urinalysis, animals were fasted at least 15 h prior to urine collection. Urine samples were stored under refrigeration until analysis. At terminal sacrifice, all rats were euthanized by exsanguination from the abdominal aorta under isoflurane anesthesia.
Hematology. Complete blood counts and hematology parameters were measured using an ADVIA 120 Hematology system. White blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular cell volume (MCV), mean corpuscular cell hemoglobin (MCH), mean corpuscular cell hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (PLT), absolute neutrophil (ANEU), absolute lymphocyte (ALYm), absolute monocyte (AMON), absolute eosinophil (AEOS), absolute basophil (ABAS), absolute large unstained cell (ALUC), absolute reticulocyte (ABET), and percent reticulocyte (% RET) were evaluated. Coagulation was determined on a Siemens Systmex CA620 automated coagulation system.

Serum chemistry. Serum sodium (Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphorus (PHOS), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), urea nitrogen (BUN), cholesterol (CHOL), creatinine (CREAT), glucose (GLU), total protein (TP), total bilirubin (TBIL), triglycerides (TRIG), sorbitol dehydrogenase (SDH), total bile acids (TBA), and globulin (GLOB) were measured on a COBAS C311 automated clinical chemistry analyzer.

Urinalysis. Urine quality, color, and clarity were evaluated visually. Other parameters, such as pH, ketone, glucose, bilirubin, specific gravity, blood, volume, protein, and urobilinogen, were measured using a Siemens Multistix SG 10, and urine sediment was evaluated microscopically.

Anatomic pathology. Necropsy included the examination of the external surface of the body, all orifices, musculoskeletal system, and the cranial, thoracic, abdominal, and pelvic cavities, including associated organs and tissues. Any gross lesions were recorded.

Histopathology. Histopathological examination was performed on the preserved organs and tissues of the animals from the 4% CC and 4% CNC groups. Any tissues and organs with macroscopic observations in other control or treatment groups were also evaluated, as were gross lesions of note in all control and test groups. The following tissues were preserved in 10% neutral buffered formalin: prostate and seminal vesicles, adrenals, aorta, bone (femur), bone marrow (from femur and sternum), brain (sections including medulla/pons, cerebellar, and cerebral cortex), cecum, cervix, colon, duodenum, esophagus, Harderian gland, heart, ileum with Peyer’s patches, jejunum, kidney, larynx, liver, lungs, lymph node mandibular, lymph node mesenteric, mammary gland, nasal turbinates, nose, ovaries, oviducts, pancreas, parathyroid, peripheral nerve (sciatic), pharynx, pituitary gland, rectum, salivary glands (sublingual, submandibular, and parotid), skeletal muscle, skin, spinal cord (cervical, mid-thoracic, and lumbar), spleen, sternum, stomach, thymus, thyroid, trachea, urinary bladder, uterus, and vagina. The epididymides, eyes, optic nerve, and testes were preserved in modified Davidson’s fixative and then stored in 70% ethanol. The fixed tissues were trimmed, processed, embedded in paraffin, sectioned with a microtome, placed on glass microscope slides, stained with hematoxylin and eosin, and examined by light microscopy slide preparation, and evaluation was conducted in compliance with GLP by a board-certified veterinary pathologist. All slides were evaluated and findings entered into Pristima® software. Each tissue was assessed for macroscopic observations, and then each tissue was assessed based on any remarkable incidences, defined as: 1 = minimal or present; 2 = mild; 3 = moderate, 4 = marked, and 5 = severe.

In vitro testing

Preparation of cellulose suspensions. Samples were weighed on an analytical balance (Mettler Toledo, Columbus, OH, USA) and diluted in ultrapure water (18.2 Qhm; MilliQ Water Purification, Millipore, Burlington, MA, USA) to produce 2% cellulose suspensions. To ensure dispersion, solutions were mixed using a Vortex Genie 2 (Scientific Industries, Bohemia, NY, USA) for 10 min prior to analyses.

Preparation of simulated gastrointestinal digested cellulose. Physical, chemical, and biological characterization of CNC and CC after simulated in vitro gastrointestinal and lysosomal digestion followed EFSA guidance. In vitro gastrointestinal digestion used physiologically relevant chemical conditions, enzymes, and salts in the mouth, stomach, and intestinal compartments, respectively [15].

The procedure outlined in Minekus et al. was followed to produce the oral phase digestate [15]. All reagents were purchased from Sigma-Aldrich (St. Louis, MI, USA) at highest purity available. Briefly, CNC and CC were suspended in salivary alpha-amylase. Calcium chloride and ultrapure water were added to achieve a final concentration 0.75 mM. The mixture was incubated for 2 min at 37 °C and then diluted by half volume with gastric fluid digestate fluid (i.e. pepsin, calcium chloride, phospholipids, and hydrochloric acid [pH-adjusted to 3.0]). Mixture was shaken and incubated again for 2 h and then diluted by half volume again with intestinal phase fluid (i.e. pancreatin, bile, calcium chloride, and sodium hydroxide [pH-adjusted to 7.0]). CNC and CC samples were incubated in the intestinal phase fluid for one of four timepoints (i.e. 15 and 30 min, 1 or 4 h). For storage, samples were stored at 4 °C for up to 2 weeks.

Preparation of simulated lysosomal digested cellulose. In vitro lysosomal digestion exposed cellulose materials to conditions within lysosomes, simulating intracellular digestion conditions. A suspension of Artificial Lysosomal Fluid (ALF) was produced to simulate lysosomal digestion of cellulose materials following the procedure outlined in Stopford et al. [16]. All reagents were purchased from Sigma-Aldrich (St. Louis, MI, USA) at highest purity available. Briefly, CNC and CC were suspended in a complex mixture of sodium chloride, sodium hydroxide, citric acid, calcium chloride, disodium phosphate, sodium sulfate, magnesium chloride, glycerol, trisodium citrate, sodium tartrate, sodium L-lactate, sodium pyruvate, and formaldehyde [16–19]. ALF was pH-adjusted to 4.5 and CNC and CC were incubated at 37 °C for one of four timepoints (i.e. 30 min; 2, 24, or 72 h). For storage, samples were stored at 4 °C for up to 2 weeks.

Physical and chemical characterization. Particle size and surface charge analysis. To determine the Hydrodynamic Diameter (HDD), dispersity index (DI), and zeta potential (ζ potential), DLS techniques were used with a Zetasizer Nanoseries Nano-ZS (Malvern Pananalytical, Almelo, Netherlands). CNC and CC suspensions were diluted to 0.01% with ultrapure water and transferred to a capillary cell DTS 1060 (Malvern Pananalytical, Almelo, Netherlands). For HDD and DI, each sample was scanned for 10 s, 11 times, and in triplicate. A 173° backscatter angle was used in general purpose mode. For ζ potential measurements, the Helmholtz–Smoluchowski model was utilized at 25 runs and in triplicate.
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Light microscopy preparation and imaging. CNC and CC suspensions were diluted to 0.01% and a droplet was allowed to dry on a glass slide. Once dried, CNC and CC samples were imaged on a stereomicroscope (Stereomicroscope SZX16 Olympus Corporation) in brightfield mode. Magnification used was 10X.

TEM preparation and imaging. CNC and CC suspensions were diluted to 0.01% before loading onto grids. Formvar carbon-coated copper grids 200 (EMS, Hatfield, PA, USA) were used. Once dried, the CNC and CC samples were imaged on a TEM JEM-1010 (JEOL Inc., Akishima, Tokyo, Japan) at an accelerating voltage of 60 kV (spot size of 2.0). Scale bars are included in each micrograph.

Scanning electron microscopy preparation, imaging, and chemical composition. Similar to TEM preparation, CNC and CC dispersions were diluted to 0.01% for before mounting onto pin stubs. Samples were imaged on a scanning electron microscope (SEM) Versa 3D, FEI Focused Ion Beam (Field Electron and Ion Company, FEI; Hillsboro, OR, USA) at an accelerating voltage of 30 kV. Energy Dispersive X-ray Spectroscopy (EDXS) during SEM imaging examined the elemental composition of CNC and CC samples (xT Microscope Control version 6.3.2). Magnification was 500X.

Metal impurities. Purity of the CNC and CC samples were assessed via ICP-MS. Prior to analysis, samples were acid-digested via nitric and hydrochloric acids in a hot block set at 95°C in a plastic digestion vessel for 1 h. Samples were then removed, allowed to cool at room temperature, and spiked with additional HNO₃ to ensure complete digestion. This process was repeated in triplicate until CNC and CC samples were clear and colorless. Samples were analyzed on an ICP-MS 7900 (Agilent, Santa Clara, CA, USA) using internal standards. Data acquisition and analysis was completed with MassHunter Software (Agilent).

Biological testing. Co-culture model assembly. Briefly, human colon carcinoma Caco-2 cells (ATCC, Manassas, VA, USA), human mucous-producing HT29-MTX cells (Sigma, St. Louis, MI, USA), and human Burkitt’s lymphoma Raji B (ATCC) cells were cultured in Dulbecco’s Modified Eagle Medium/F-12 (DMEM/F12; 1:1), fetal bovine serum, 10 000 U/mL penicillin, and 10 mg/mL streptomycin (MP Biomedicals, Santa Ana, CA, USA), trypsin–EDTA (Invitrogen, Waltham, MA, USA). Transwell® polycarbonate inserts (12 wells, pore diameter of 4-μm polycarbonate; Corning, NY, USA) were used in model assembly. Raji B, Caco-2, and HT29-MTX cells were seeded at a ratio of 9:9:1, with the HT29-MTX and Caco-2 cells in the apical chamber of Transwell® inserts and Raji B cells in the basolateral side. Cell culture medium was exchanged every other day.

Culture exposure to cellulose materials. Cells were exposed to CNC or CC by resuspending pristine samples in ultrapure water (0.02%) or using digested samples in neutralized fluid (0.02%). CNC or CC was added into the apical chamber of Transwell® inserts and the system was incubated for 1, 6, 24, or 48 h in a 37°C incubator under 5% CO₂ atmosphere.

Following incubation, the co-culture was washed with PBS solution, replaced with fresh media, and assessed for cellular responses via metabolic activity, gastrointestinal barrier integrity impairment, oxidative stress, and proinflammatory response. Comparison groups include negative control treatments (untreated), vehicle control treatments (either deionized water or simulated gastrointestinal fluid), and positive control treatments (rotenone, lipopolysaccharides, and hydrogen peroxide).

Cytotoxicity/cell viability. Metabolic activity was measured using the MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] assay (MTS, Madison, WI, USA). MTS solution was added to each exposed co-culture per manufacturer’s direction [20, 21]. After 2-h incubation, the absorbance was measured at 490 nm on a plate reader (Synergy, BioTek, Winooski, VT, USA).

Gastrointestinal barrier integrity impairment. Transepithelial/Transendothelial Electrical Resistance (TEER) was used as an indicator of membrane integrity and permeability using a volt-meter (ZVOM2, Epithelial Voltohmeter, World Precision Instruments, Inc., Sarasota, FL, USA) [22]. Briefly, transwell inserts were connected to voltmeter electrodes and resistance was recorded. Any samples exhibiting initial TEER values less than or equal to 190 U were excluded [23]. TEER was measured twice daily for 7 days in triplicate [24].

Pro-inflammatory response markers. The inflammatory response marker interleukin 6 (IL-6) was measured using an Enzyme-linked immunosorbent Assay (Invitrogen, Carlsbad, CA, USA). Exposed cells were washed with PBS and lysed with Radio-Immunoprecipitation Assay Buffer (Invitrogen). Lysate was collected, centrifuged at 12 000 rpm, and plated per manufacturer’s direction. Absorbance was read on a plate reader (Synergy H1, BioTek) at 450 nm (620 nm as a reference wavelength) [25, 26].

Oxidative stress. For flow cytometry, cells were harvested from co-cultures previously exposed to pristine or digested CNC or CC and were spiked with 0.5 μM CellROX Green (ex/em: 508/525 nm; ThermoFisher) or 0.5 μM SyTox Red (ex/em: 640/658 nm; ThermoFisher) in 96-well V-bottom plates. After 37°C incubation for 30 min, samples were analyzed within on a FACSCoupe flow cytometer (BD Biosciences, San Jose, CA, USA). Flow rate was set to 3.0 μL/s with a 150-μL injection volume, 100-μL mixing volume, 250-μL/s mixing speed, and 800-μL wash volume. For each sample, 50 000–200 000 events were collected.

Statistical analyses

In vivo experiments. To compare control and test groups, parametric one-way analyses of variance (ANOVA) were performed for each quantitative parameter with homogeneous variance (assessed using Levene’s test). Welch’s ANOVAs were performed for parameters violating the homogeneity of variance assumption. Separate analyses were performed on the data collected for males and females. Parameters found significant in the ANOVA were further analyzed using post hoc Tukey’s tests (for parameters with homogenous variance) or Games-Howell tests (for parameters with unequal variance). Differences were considered significant at P < 0.05. All statistical analyses were performed using RStudio (Version 1.2.1335).

In vitro experiments. For each data set, a two-way ANOVA (alpha = 0.05) was performed [27]. ANOVA was also implemented to confirm the parametric two-way ANOVA results [28]. In order to confirm assessment, Tukey’s Honest Significant Differences was conducted. All statistical analyses were performed in Prism 8.3.0.0 (GraphPad, San Diego, CA, USA).

Results

Physical and chemical characterization

Light (Fig. 2) and electron micrographs (Fig. 3) show the general morphology of CNC and CC. CNC is rod shaped, with individual crystals having widths <10 nm and lengths that vary from 25 to 250 nm. DLS measurements report an average HDD of...
has a near neutral EDXS demonstrates the similar elemental composition of CNC because both materials are derived from cellulosic feedstock. CNC has significant changes to zeta potential following simulated gastrointestinal, nor lysosomal digestion (Fig. 4A–D). CC has an average HDD of 26.7 ± 6 μm and DI of 0.65 ± 0.9 mV. Neither CNC nor CC changed the DI of CNC at any timepoint examined. In comparison, there were no adverse clinical observations for significant periods of time in control or test animals.

Clinical observations. There were no treatment-related mortalities attributed to the consumption of CNC or CC at any concentration. One rat in the 4% CC group experienced self-inflicted mechanical damage and was humanely sacrificed on Day 66. There were no treatment-related mortali-
ities attributed to the diet.

Body weight, food consumption, body weight gain, organ weight. There were no differences in body weight parameters between the 2, 3, and 4% male and the 2 and 3% female CNC and CC groups (P > 0.05) (Fig. 6). Females fed 4% CNC weighed 400.3 ± 51.2 g on Day 90 and were significantly heavier than females fed 4% CC, weighing 332.8 ± 44.7 g (Fig. 6). Food consumption was higher in the 4% CNC female group (149.9 ± 19.6 g/day) compared with the 4% CC group (127.4 ± 18.4 g/day) (P < 0.05). Females fed 4% CNC on average gained 2.45 ± 0.51 g/day, and those fed 4% CC gained 1.73 ± 0.43 g/day (P < 0.05). There were no significant differences (P > 0.05) in organ weight between male 2, 3, and 4%, and female 2 and 3% CC and CNC groups (Tables 1 and 2). The heart, kidney, and spleen were significantly heavier in the 4% CNC group compared with the 4% CC group; however, no-dose-related trends were observed. The relative organ weights, as compared with total body weight, were not significantly different between any of the CC and CNC groups.

Daily dietary intake. There were no differences in daily dietary intake of cellulose (mg/kg/day) in male or female rats between control and treatment groups (P > 0.05) (Table 3).

Optohamology. All animals in the CNC treatment groups were considered normal. One female rodent exposed to 2% CC had extensive chorioretinal scarring in the left eye. Another female rodent exposed to 3% CC female had chorioretinal scarring in the right eye. As isolated incidents, these observations were not considered attributable to the diet.

Clinical pathology. The clinical pathology investigations indicate no toxico logically significant results (P > 0.05). There were no other significant adverse CNC effects on the hematology factors (Supplemental Table 1), the serum chemistry (Supplemental Table 2), or the urine parameters (not shown).
Hematology, coagulation, and serum chemistry values fall within normal range of Sprague–Dawley rats fed similar diets [29, 30]. There were neither adverse effects nor lesions observed during the gross necropsy.

**Histopathology.** Vacuolation of periportal hepatocytes (variably-sized, clear cytoplasmic vacuoles) in the liver was present in both 4% CC and 4% CNC groups. There was little variation in incidence and severity between the groups, and vacuolation was not accompanied by hepatocyte degeneration or any other pathologic observations in the liver. There were no other consistent macroscopic or microscopic adverse effects observed. Without any other apparent altered liver function or clinical histopathology observations, this is not considered to be an adverse result. All macroscopic observations were isolated and considered to be common background changes commonly noted in laboratory rats, with no relation to dietary test substance administration.

**In vitro testing**

**Cytotoxicity/cell viability.** Metabolic activity of the intestinal co-culture, as a measure of cell viability, is measured with the MTS assay following exposure to pristine or digested cellulose for 1, 6, 24, or 48 h (Fig. 7). Similar responses in co-cultures exposed to the negative control treatment (media only), deionized water (vehicle control treatment for pristine cellulose materials), and simulated gastrointestinal fluid (vehicle control for digested cellulose materials) for 1, 6, 24, and 48 h are observed and represent the metabolic activity of healthy cell cultures (Fig. 7A). Significantly elevated metabolic activity is observed for all timepoints...
Figure 5: CNC and CC are derivatives of cellulose and composed primarily of carbon and oxygen. Energy dispersive X-ray spectroscopy of (A) CNC and (B) CC.

Figure 6: Overall and mean weekly body weights of rats consuming either CNC or CC on Day 90 at 2, 3, or 4% diet. Average male (A) and female (B) rat body weight, Day 0–90.

following exposure to the positive control treatment (50-μM Rotenone; Fig. 7A).

Exposure to CNC or CC, in either pristine or digested form, has no significant impact on the metabolic activity of the intestinal co-culture. All cellulose material exposures demonstrate a trend of increasing MTS absorbance over time (1–48 h) anticipated with viable growth of the co-culture. There are no significant differences in metabolic activity between cells exposed to pristine...
Table 1: Mean organ weights in male rats on Day 90

| Organ weight (g) | Males | CNC | 2% | 3% | 4% | 2% | 3% | 4% |
|-----------------|-------|-----|----|----|----|----|----|----|
| Adrenal glands  | 0.0915| 0.0655| 0.0601| 0.0581| 0.0607| 0.0652|
| Brain           | 2.317 | 2.237| 2.332| 2.310| 2.327| 2.330|
| Epididymides    | 1.5952| 1.6097| 1.6159| 1.6591| 1.5047| 1.5273|
| Heart           | 1.606 | 1.604| 1.637| 1.610| 1.588| 1.623|
| Kidneys         | 3.611 | 3.669| 3.722| 3.725| 3.295| 3.472|
| Liver           | 15.022| 14.933| 16.574| 15.306| 14.022| 14.799|
| Spleen          | 0.950 | 0.948| 1.016| 0.957| 0.990| 0.958|
| Testes          | 3.575 | 3.547| 3.723| 3.693| 3.540| 3.492|
| Thymus          | 0.2762| 0.2979| 0.3007| 0.3379| 0.2845| 0.3162|

Table 2: Mean organ weights in female rats on Day 90

| Organ weight (g) | Females | CNC | 2% | 3% | 4% | 2% | 3% | 4% |
|-----------------|---------|-----|----|----|----|----|----|----|
| Adrenal glands  | 0.0713 | 0.0694| 0.0720| 0.0675| 0.0584| 0.0723|
| Brain           | 2.100 | 2.118| 2.093| 2.087| 2.135| 2.088|
| Heart           | 1.119 | 1.082| 1.083| 1.058| 1.064| 1.233+|
| Kidneys         | 2.431 | 2.340| 2.207| 2.142| 2.222| 2.637+|
| Liver           | 10.109| 9.675| 9.603| 9.283| 9.422| 11.178|
| Ovaries with oviducts | 0.1243 | 0.1122| 0.1204| 0.1271| 0.1217| 0.1092|
| Spleen          | 0.629 | 0.597| 0.539| 0.584| 0.568| 0.658+|
| Thymus          | 0.3017| 0.3085| 0.2581| 0.2935| 0.3009| 0.3136|
| Uterus          | 0.749 | 0.827| 0.844| 0.654| 0.761| 0.773|

*P < 0.05 in comparison to corresponding CC group.

Table 3: Daily dietary intake of cellulose (mg cellulose/kg rat/day ± SD)

| Cellulose intake (mg/kg/day) | Males | Females |
|-----------------------------|-------|---------|
| 2% CC                       | 1070.4 ± 48.3 | 1311.7 ± 52.3 |
| 2% CNC                      | 1056.0 ± 23.9 | 1278.2 ± 52.9 |
| 3% CC                       | 1535.8 ± 54.8 | 1920.2 ± 119.5 |
| 3% CNC                      | 1583.7 ± 77.2 | 1929.8 ± 99.2 |
| 4% CC                       | 2119.2 ± 128.9 | 2597.5 ± 135.3 |
| 4% CNC                      | 2085.3 ± 118.8 | 2682.8 ± 93.1 |

or digested forms of either CNC or CC and their corresponding vehicle controls. Furthermore, simulated digestion of CNC and CC does not impact the metabolic response of cells exposed in vitro. No significant differences are observed in the metabolic activity of co-cultures exposed to pristine versus digested forms of CNC (Fig. 7B) or CC (Fig. 7C), for all timepoints.

Gastrointestinal barrier integrity impairment. Similar responses are observed in TEER measurements of co-cultures exposed to negative (untreated) and vehicle (deionized water; simulated gastrointestinal fluid) control treatments (Fig. 8A). Co-cultures show daily fluctuations between morning and evening measurements with an increase in resistance observed over time associated with normal culture growth and maintained epithelial structure. In contrast, co-cultures exposed to rotenone do not show increasing TEER over time; this observation is indicative of cell death and loss of epithelial structure (Fig. 8A).

A temporary decrease in TEER is observed following exposure to CNC and CC materials (Day 2 PM). This observed decrease in electrical resistance is associated with addition of cellulose material, which momentarily interferes with the electrical resistance measurement but is not reflective of changes in cell co-culture barrier integrity (Fig. 8B). Similar to negative and vehicle controls, cellulose exposure does not interfere with co-culture barrier integrity, and by 7 days, overall TEER of 150–200 Ωcm² is achieved.

Proinflammatory response markers. IL-6 expression is used as a marker of potential proinflammatory responses. Figure 9A shows IL-6 expression in co-cultures exposed to control treatments including negative controls (untreated cells), vehicle controls (deionized water and simulated GI fluid), and positive controls (LPS). Results show minimal expression of IL-6 in negative and vehicle control treatments for all post-exposure timepoints examined, with lowest overall levels observed 24 and 48 h post-exposure. Exposure to LPS caused a significantly elevated expression of IL-6 that is sustained for 48 h. Exposure to either pristine or digested CNC (Fig. 9B) and CC (Fig. 9C) for 1, 6, 24, or 48 h had minimal proinflammatory responses, with similar IL-6 expression to negative and vehicle control treatments.
IL-6 expression was greatest immediately following exposure to CNC and CC (1 h) and decreased over time (6, 24, and 48 h). One exception was found following 48 h exposure to pristine CC, where significantly elevated IL-6 expression was observed compared with vehicle control; however, IL-6 expression was still below 1 pg/mL (verified with a standard curve) and insignificant compared with the positive control treatment. There were no differences observed in the proinflammatory responses of cells exposed to either pristine or digested forms of cellulose materials.

Oxidative stress. Exposure to either pristine or digested CNC or CC did not result in a significant elevation of oxidative stress for any post-exposure timepoints examined (Fig. 10). Experimental control groups are shown in Fig. 10A, with over 97% of cells exposed to negative and vehicle control treatments staining as “alive with no oxidative stress” for all timepoints examined. In contrast, in cells exposed to hydrogen peroxide (positive control), over 80% of the cell population stained as “alive but oxidatively stressed.” Cells exposed to either pristine or digested forms of CNC (Fig. 10B) or CC (Fig. 10C) did have neither a significant induction of oxidative stress nor a decline in cell viability. Similar to negative and vehicle control treatments, greater than 97% of cells exposed to CNC or CC stained as “alive with no oxidative stress,” for all timepoints examined. Furthermore, no significant
Figure 9: Intestinal co-culture exposure to either pristine or digested CNC or CC had minimal proinflammatory response. IL-6 release in gastrointestinal co-culture exposed to (A) controls [untreated cells, deionized water (vehicle control for pristine cellulose materials), simulated gastrointestinal fluid (vehicle control for digested cellulose materials), and 50-μM lipopolysaccharides (LPS; positive control)]. Statistical significance is observed between each vehicle control and LPS at each timepoint tested (*P < 0.05). MTS absorbance in gastrointestinal co-culture exposed to pristine digested pristine and digested (B) CNC or (C) cellulose. No statistical significance was observed between CNC or CC exposed cells versus vehicle controls. Data for digestion time period of 4 h are shown.

Differences are observed in either cell viability or oxidative stress for cells exposed to pristine versus digested cellulose materials.

Discussion

Cellulose, a fundamental structural component of plants, is an important dietary fiber that has been safely added to food for decades. Several studies and scientific reviews have concluded that cellulose, and many of its derivatives, is safe for use in foods ([3], [31, 32]) and has been authorized as food additives in several markets worldwide including the USA, Canada, and the European Union. Sulfated CNC is a novel form of cellulose with several unique properties being developed for a variety of applications in the food industry; therefore, a proactive demonstration of safety is required to promote safe and responsible commercialization. This study compares the physical, chemical, and toxicological properties of CNC alongside a conventional form of cellulose that has been used in food for over 85 years (CC; Solka Floc®).
Figure 10: Intestinal co-cultures exposed to either CNC or CC, in pristine or digested form, did not induce oxidative stress or declines in viability. Oxidative stress and viability measured in gastrointestinal co-culture exposed to (A) controls [untreated cells, deionized water (vehicle control for pristine cellulose materials), simulated gastrointestinal fluid (vehicle control for digested cellulose materials), and 100-mM hydrogen peroxide (H$_2$O$_2$; positive control)]; and pristine and digested (B) CNC or (C) cellulose. Data for digestion time period of 4 h are shown.

The testing strategy adopts both traditional (i.e. animal testing) and next-generation (i.e. simulated digestion and in vitro testing) testing strategies as part of the safety demonstration of CNC for food and food-related applications and drew upon the latest guidance for demonstrating the safety of nanomaterials in food and feed [11].

Physical and chemical characterization of pristine CNC and CC demonstrate that these materials have the same fundamental molecular composition, both being derived from cellulose; however, there are significant physical and chemical differences between these two forms of cellulose. CC is over a magnitude of order larger than CNC, containing particles in the micron range. In comparison, CNC is composed of particles that are generally 100’s of nanometers in length and <10 nanometers in width. In addition, CNC production introduces sulfate-group functionalization resulting in a more negative surface charge compared with CC which is not functionalized.

Simulated gastrointestinal and lysosomal digestion of CNC and CC did not result in any significant changes to their physical or chemical characteristics. Generally, the HDDs, DIs, and zeta potentials of digested CNC and CC (both gastrointestinal and lysosomal) were similar to their pristine forms, for all time-points examined. This suggests that both CNC and CC remain relatively unchanged physically and chemically as they undergo digestion, in line with our current understanding of cellulose metabolism in the human gastrointestinal tract. Humans lack
digestive enzymes capable of breaking down cellulose; several absorption, distribution, metabolism, and excretion studies have shown that it is generally 100% excreted in the feces [32].

The physical and chemical testing strategy employed here is based on guidance released by EFSA to demonstrate the safety of novel materials in food and feed. However, two aspects of the guidance created challenges for carbon-based materials such as cellulose. EFSA recommends characterizing the morphology of materials post-digestion with electron microscopy as well as assessing the potential for dissolution using ICP-MS or related techniques. However, both CNC and CC are carbon-based, making these characterizations challenging or simply not possible. Both CNC and CC proved difficult to detect in the complex digestion mixtures using electron microscopy. Furthermore, dissolution is commonly characterized by measuring an increase in ionic species over time using ICP-MS (e.g. the dissolution of silver measured with an increase in Ag+ over time); this was not possible for either CNC or CC since ICP-MS cannot detect ionic carbon. Given that some of the most promising nanomaterials coming to market are carbon-based (e.g. cellulose nanomaterials, graphene nanomaterials, and carbon nanotubes), this limitation could be addressed by EFSA in their guidance. However, as stated earlier, neither dissolution nor any change in morphology of these materials had been anticipated post-digestion, given that humans lack digestive enzymes capable of breaking these materials down.

Although several physical and chemical differences exist between CNC and CC, both of these materials were hypothesized to behave similarly biologically. The in vivo and in vitro toxicity assessments support this hypothesis. Traditionally, 90-day dietary animal testing is used to demonstrate the safety of food additives and gain regulatory authorization in key markets worldwide. Two range-finding pilot studies (based on OECD TG 407) determined that there were no adverse effects associated with feeding 5% CNC over 7 days or with feeding up to 1.2% CNC over 14 days. The 90-day subchronic feeding study in rats found that, similar to conventional forms of cellulose such as Solka Floc, 90-day consumption of a diet composed of 2, 3, or 4% CNC did not result in any treatment-related adverse effects. Effects assessed included mortality, clinical symptoms, body weight, food consumption, food efficiency, ophthalmology, hematology, serum chemistry, urinalysis, anatomic pathology, organ weight, and histopathology. The study design began in April 2018 and therefore was conducted according to OECD TG 408 [14]. In June 2018, the test guideline was updated (OECD TG 408; 2018) to include additional endocrine-sensitive endpoints, which were not evaluated in the current study. However, there are no indications from any publicly available studies that exposure to dietary celluloses results in any negative endocrine effects that would warrant measurement [11].

The rats fed 4% CNC for 90 days consumed significantly more food than rats fed 4% CC, and as a result, gained more weight. This increased intake was not associated with any adverse clinical or pathological effects and may indicate increased palatability, though no significant weight differences were observed between other groups. Under the conditions of the subchronic study and based on the toxicological endpoints evaluated, the calculated no-observed-adverse-effect level (NOAEL) for CNC is 2085.3 (males) and 2682.8 (females) mg/kg/day and for CC is 2119.2 (males) and 2597.5 (females) mg/kg/day. To date, only two other studies have evaluated the oral toxicity of CNC using standardized in vivo testing and found no adverse effects from consumption. O’Connor et al. [33] reported no adverse effects from one-time oral exposure to CNC in rats following OECD TG 425 and calculated a LD50 greater than 2000 mg/kg bw. Similarly, O’Connor et al., [33] completed a 28-day repeated dose oral toxicity assessment of CNC in rats following OECD TG 407 and found no adverse effects and calculated a NOAEL greater than 2000 mg/kg bw.

To our knowledge, no studies to date have evaluated potential impacts from CNC exposure on the microbiome in vivo. However, it is hypothesized that CNC will behave similarly to other forms of cellulose, such as cellulose nanofibrils (CNFs) [34], and found that rats gavaged with 1% CNF for 5 weeks did not have any substantial effects on the fecal metabolome but did have noted changes in microbial diversity, cytokine production, and epithelial cell junction gene expression. However, these perturbations were minor and not associated with any pathological effects. It is well established the dietary fibers (and other dietary components) that will alter microbiota in the gut by affecting bacterial fermentation, colony size, and species composition (e.g. [35]). There are no indications in the literature that CNC exposure will result in any adverse effects related to the microbiome. As part of the safety demonstration for CNC, additional toxicology studies in vitro were commissioned following guidance released by EFSA for screening level testing of novel food ingredients. The aim was to provide additional data demonstrating the biological similarity of CNC to conventional forms of cellulose in the human gut and further promote the use of alternative testing strategies in the safety evaluation of new chemicals. Both CNC and CC, in their pristine form as well as following simulated gastrointestinal digestion, did not induce any adverse effects in the intestinal co-culture model up to 0.4% by weight. Exposure to either form of cellulose did not induce any significant cytotoxicity; impairment to the epithelial barrier integrity; oxidative stress; or inflammatory response. To our knowledge, only one other study has evaluated the oral toxicity of CNC using in vitro models. DeLoid et al. [36] exposed CNC to a simulated gastrointestinal digestion mimicking conditions encountered in the mouth, stomach, and intestinal compartments. Digested CNC was subsequently exposed to an intestinal co-culture model up to 1.5% w/w. Similar to our findings [36], it did not find any significant changes in cytotoxicity, Reactive Oxygen Species (ROS), or monolayer integrity following exposure to digested CNC; although a slight increase in ROS, production was observed when CNC was digested in a fasting model. The authors concluded that CNC has little acute toxicity and is safe for oral consumption up to 1.5% w/w.

Despite the physical and chemical differences that exist between CNC and CC (e.g. size, shape, surface charge, and surface chemistry), these materials behave similarly biologically and have similarly low toxicity in vivo and in vitro. However, the physical and chemical characteristics of nanomaterials have been shown to impact their toxicity, and the relationships between physical and chemical characteristics to toxicological outcomes (known as structure–activity relationships) are still not fully understood [37]. The results presented here are for one form of sulfated CNC produced by InnoTech Alberta. CNC produced by other manufacturing methods, from other fiber sources, or with significant changes to their physical or chemical properties may impact their toxicity and ultimately safety for food-related applications. The in vitro testing strategy described here is a useful screening tool to evaluate the impact of different physical and chemical characteristics on toxicological outcomes of future forms of CNC without further animal testing.
Conclusion

Although some physical and chemical differences exist between CNC and CC, these materials are both derived from cellulose and behave similarly in oral in vivo and in vitro studies examining their safety. The 90-day subchronic toxicity test found no adverse effects from oral CNC or CC exposure in rats up to 4% of the diet. Simulated in vitro digestion of CNC and CC found that these materials remain relatively physically and chemically unchanged following conditions that mimic gastrointestinal and lysosomal digestion. Lastly, neither CNC nor CC had any significant adverse effects on an in vitro intestinal co-culture, in either their pristine or digested form. These results are in line with previous in vivo and in vitro studies demonstrating the safety of CNC for food-related applications. Altogether, these data demonstrate that CNC produced by InnoTech Alberta has a similar toxicological profile as CC in the gastrointestinal tract. Given the long history of safe use of CC as a food additive, we conclude that CNC raises no safety concerns when used as a food ingredient and has a NOAEL of 2085.3 (males) and 2682.8 (females) mg/kg/day. The in vitro testing strategy described here is a useful tool to evaluate the impact of physical or chemical changes to CNC on oral toxicity as future commercial forms are developed and tailored to specific uses in food.

Supplementary data

Supplementary data is available at TOXRES Journal online.

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Conflict of interest statement.

The authors have no conflicts of interest to declare.

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