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Toxicological effects of ingested nanocellulose in in vitro intestinal epithelium and in vivo rat models

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Abstract

Cellulose is widely used as a thickener and filler in foods and drugs. It has been designated "generally regarded as safe" (GRAS). Nanocellulose (NC) has many additional potential applications designed to improve food quality and safety, but has not yet been designated as GRAS. Here we present results of toxicological studies of ingested NC in physiologically relevant *in vitro* and *in vivo* systems. *In vitro* studies employed a gastrointestinal tract simulator to digest two widely-used forms of NC, nanocellulose fibrils (CNF) and cellulose nanocrystals (CNC), at 0.75 and 1.5% w/w, in a fasting diet as well as in a standardized food model based on the average American diet. A triculture model of small intestinal epithelium was used to assess effects of a 24-hour incubation with the digested products (digesta) on cell layer integrity, cytotoxicity and oxidative stress. Other than a 10% increase over controls in reactive oxygen species (ROS) production with 1.5% w/w CNC, no significant changes in cytotoxicity, ROS or monolayer integrity were observed. *In vivo* toxicity was evaluated in rats gavaged twice weekly for five weeks with 1% w/w suspensions of CNF in either water or cream. Blood, serum, lung, liver, kidney, and small intestine were collected for analysis. No significant differences in hematology, serum markers or histology were observed between controls and rats given CNF suspensions. These findings suggest that ingested NC has little acute toxicity, and is likely non-hazardous when ingested in small quantities. Additional chronic feeding studies are required to assess long term effects, and potential detrimental effects on the gut microbiome and absorbance of essential micronutrients. These studies are underway, and their outcome will be reported in the near future.

Introduction

Engineered nanomaterials (ENMs) are often added to foods in order to improve quality, safety, or nutrition.[1–14] Nanocellulose (NC), derived from natural wood fiber or other plant

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sources, could be used in food packaging to help increase shelf life, or incorporated into the food, where it may serve as a thickener or binder (superior to its micron-scale counterpart). It could also be included as a non-caloric fiber source, used to provide stabilization for foams (e.g., whipped toppings) or emulsions (e.g., salad dressings), incorporated in ground meats to help retain moisture during cooking, added to bread dough to increase bulk and improve texture of the baked product, or used in other foods to improve appearance and mouth feel.[3,4,10,15–17] In addition to improving food quality and appeal, ingested nanocellulose, either incorporated into foods or ingested separately, may have applications in favorably modulating digestion and absorption of nutrients. We have recently shown that incorporating small amounts of cellulose nanofibrils (CNF) in a high fat diet reduces hydrolysis of triglycerides and release of free fatty acids in the small intestine, resulting in reduced absorption of fat[18].

For decades, micron-scale cellulose and its derivatives (e.g., methylcellulose) have been added to processed foods as fillers and thickeners, and have been designated “generally regarded as safe (GRAS)” for ingestion. However, NC has yet to be accepted as safe or approved as a food ingredient. This is primarily due to the well-known fact that nano-scale materials often exhibit very different properties and interactions with biological systems compared with their micron and larger-scale counterparts.[19–26] Thus, investigating the toxicology of ingested nanomaterials is of great importance. Several studies have shown that ingested inorganic nanoparticles can damage the intestinal microvilli and limit nutrient absorption.[27–30] Moreover, inorganic ENMs such as TiO_2, Ag and ZnO have been linked to tumorigenesis, genotoxicity, changes in the gut microbiome and exacerbation of inflammatory diseases such as colitis.[31–36]

Studies of nanocellulose exposure in various *in vitro* cell lines and animal models are limited and have found no evidence of significant toxicity[37–39], however these studies have either focused on other routes of exposure (e.g., inhalation), or have ignored the effects of digestion and interactions with the food matrix on the physicochemical properties of ingested NC. Yet understanding and accounting for these transformations are essential to the study of ingested nanomaterials[40–42]. Any meaningful *in vitro* study of ingested NC toxicity must include a process for recapitulating the effects of digestion, and include the influence of the food matrix.

Here we report results of *in vitro* cellular studies using an integrated methodology recently developed by the authors which takes into consideration the effects of the food matrix and digestive process[42]. We also present acute *in vivo* animal studies to assess the potential toxicity of nanocellulose. In the *in vitro* studies, a three-phase (mouth, stomach, small intestine) gastrointestinal tract simulator[42] was used to subject NC (both nanocellulose fibrils or CNF and cellulose nanocrystals or CNC) to the chemical and physical conditions of the GI tract[18,42]. The resulting digested products (digesta) were applied to a triculture model that includes enterocytes, goblet cells and M-cells to emulate the intestinal epithelium (Figure 1), previously described and characterized elsewhere[18,42]. After 24 hours cell layer integrity was assessed by measuring trans-epithelial electrical resistance (TEER) to evaluate the integrity of tight junctions, lactate dehydrogenase (LDH) release was measured to assess cell injury and death, and a fluorometric assay was used to measure reactive oxygen species

(ROS) production and oxidative stress. To assess toxicity *in vivo*, an acute rat gavage model was used, exposing rats twice a week for five weeks to ingested nanocellulose alone or in a food matrix. Blood, serum and tissues were collected for analysis of hematology, serum markers of disease, and histopathology, respectively. As detailed below, neither *in vitro* nor *in vivo* assessment revealed any significant toxicity. Although studies to evaluate other endpoints, such as effects on the gut microbiome and micronutrient absorbance, are needed, and potential effects of long term and possibly life-long exposure must be assessed with chronic feeding studies, these findings support the idea that ingested nanocellulose is non-toxic and likely poses little risk when ingested in small quantities. This is an important step toward acceptance of ingested NC as safe, which in turn would enable food manufacturers to utilize this material for benefits already known, and allow development of other potential important applications for these materials.

Materials and methods

Nanomaterials

The ENMs used in this study were synthesized and characterized extensively, and are part of the Harvard-NIEHS Reference ENM repository established at part of the National Institute of Environmental Health Sciences (NIEHS) Nanotechnology Health Implication Research (NHIR) Consortium. Details of the methods used to synthesize and characterize the cellulose materials used in this study were previously described in detail[43]. Fibrillar cellulose materials (CNFs), cellulose nanofibrils with an average fibril diameter of approximately 50 nm (CNF-50), and a micron scale fibrillar cellulose (MC), were created as previously described by mechanical grinding of dried sheets of softwood bleached kraft fiber (St. Felicien Mill, Canada). CNC with an average diameter of approximately 25 nm (CNC-25) was synthesized by milling the same material in the presence of sulfuric acid. Stock CNF-50 and MC contained 2.5% w/w cellulose and stock CNC-25 contained 7.0% w/w cellulose. The remainder of each stock material consisted of sterile deionized water. Methods for characterization of food-grade TiO_2 ENMs (E171), which were used as controls in the *in vitro* studies, and their physico-chemical characterization, including Brunauer–Emmett–Teller (BET), X-Ray Diffraction (XRD), TEM and SEM, were previously described in detail[44]. ENMs used in this study were characterized using the following analytical methods:

Endotoxin and sterility assessment of ENMs—ENMs were tested for endotoxin levels using the EndoZyme® recombinant factor C (rFC) assay (Hyglos, Germany) according to manufacturer's instructions as described in detail by the authors[43]. In Brief, 10 μg/mL suspensions of ENMs as well as endotoxin standard dilutions and ENM suspensions spiked with 0.5 EU/ml endotoxin were prepared in endotoxin-free water. Samples, spiked samples and standard dilutions were dispensed into a pre-warmed (37C) 96 well plate (100 μl/well), and mixed with 100 μl assay reagent (8:1:1 ratio of assay buffer, enzyme, and substrate). Fluorescence (Ex 380, Em 440) was measured at t=0 and at 90 minutes. Endotoxin levels were calculated from sample fluorescence using a standard curve equation generated from a range of endotoxin dilutions.

Microbiological sterility of all nanomaterials used was assessed using WHO protocol in the international pharmacopoeia[45] as previously described[43,46]. Briefly, materials were suspended at 1 mg/mL, and 1 mL of the suspensions were added to 10 mL of fluid thioglycolate medium at pH 6.9–7.3. The solutions were incubated at 37 °C for 14 days and examined each day for indications of bacterial growth. Every third day during incubation samples of broth were spread onto tryptic soy agar plates, and mixed with potato dextrose agar and plate count agar to create pour plates. All plates were incubated at 37 °C for 3 days and examined for growth of bacterial and fungal colonies.

Standardized food model based on the American diet

A standardized food model (SFM), based on the average American diet, was recently developed and its formulation and characterization in detail[47]. The standardized food model (SFM) is prepared as an oil-in-water emulsion consisting of 3.4% protein (sodium caseinate), 4.6% sugar (sucrose), 5.2% digestible carbohydrate (modified corn starch), 0.7% dietary fiber (pectin), 3.4% fat (corn oil), and 0.5% sodium chloride, and spray-dried to produce a powder for storage at 4°C. To reconstitute the food into liquid form, 0.67 g of the powder was combined with 3.08 g distilled water and stirred at 900 RPM for 30 minutes with an overhead stirrer.

In vitro simulated digestion.

In vitro simulated digestion was performed using a 3-phase (mouth, stomach, small intestine) simulator as previously described.[42] Nanomaterials (CNF-50, CNC-25, MC, or TiO_2 E171) were added to either a fasting food model (5 mM phosphate buffer) or standardized food model at 0.75 or 1.5 % w/w. The concentrations of nanomaterials used were chosen based on existing U.S. regulations when available, or likely usage. The GRAS material TiO_2 is allowed as a coloring agent at up to 1% by weight, per U.S. Food and Drug Administration CFR Title 21 (https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=73.575). CNC and CNF have not been approved as food additives, and therefore no guidelines regulating its concentration exist. Micron or larger scale versions of cellulose are approved GRAS food additives, with powdered cellulose allowed at up to 3.5%, and microcrystalline cellulose at up to 3.0% in some meat and poultry products by the U.S. Department of Agriculture Food Safety and Inspection Service (https://www.fsis.usda.gov/wps/wcm/connect/f547732e-3a2b-4593-b399-abb57b1e5528/binders.pdf?MOD=AJPERES). However, given the likely greater specific surface areas of their nanoscale counterparts, the high viscosities of such NC materials, particularly CNF, in suspensions greater than 1.0% by weight, and the reported efficacy in improving properties of foams, emulsions, breads and ground meat products at concentrations ranging from 0.2 to 1.0% by weight[4], we reasoned that realistic usages would be unlikely to exceed 1% by weight. Concentrations of 1.5 and 0.75% for in vitro studies were thus selected to bracket the 1% value. Food-nanomaterial mixtures were vortexed for 30 seconds to mix, and pre-warmed to 37 °C in a water bath. These mixtures were then combined with an equal volume of pre-warmed (37 °C) simulated saliva fluid, mixed by inverting for 10 seconds. The resultant mouth digesta was then combined with a simulated gastric fluid and incubated for two hours at 37 °C on an orbital shaker at 100 rpm to complete the stomach phase. In the small intestinal phase, the stomach digesta was combined with additional salts, bile extract

and lipase to simulate intestinal fluid, and incubated at 37 °C for 2 hours while maintaining a constant pH of 7.0. The pH was maintained using a TitroLine 7000 pH Stat titration device (SI Analytics, GmbH, Germany) to add 0.25 M sodium hydroxide as needed throughout the small intestinal digestion.

In vitro toxicity assessment.

Cytotoxicity was assessed as described below using a triculture model of the small intestinal epithelium grown on transwell inserts or in 96-well plates[42].

Triculture *in vitro* model—The development and methods for generating the triculture model were previously described.[42] Caco-2, HT-29MTX, and Raji B cells were obtained from Sigma-Aldrich Corp (St. Louis, MO). Caco-2 and HT29-MTX cells were grown in high-glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, cat. no. 12306c), 10 mM HEPES buffer, 100 IU/ml Penicillin, 100 μg/ml Streptomycin and non-essential amino acids (1/100 dilution of 100 X solution, ThermoFisher, Waltham, MA). Raji B cells were cultured in RPMI 1640 media supplemented with 10% FBS, 10 mM HEPES buffer, 100 IU/ml Penicillin and 100 μg/ml Streptomycin. For transwell inserts, Caco-2 and HT-29MTX cells were trypsinized and resuspended in DMEM media at 3×10^5 cells/cm^3, and combined in a ratio of 3:1 (Caco-2:HT29-MTX). 1.5 ml of the cell mixture was seeded in the apical chamber, and 2.5 ml of complete DMEM media was added to the basolateral compartment of a 6 well transwell plate (Corning, Corning, NY). Media was changed after four days and subsequently every other day until day 15. On day 15 and 16 the media in the basolateral compartment was replaced with 2.5ml of a suspension of Raji B cells at a concentration of 1×10^6 cells/ml in 1:1 DMEM: RPMI complete media. Transepithelial electrical resistance (TEER) was measured using an EVOM2 Epithelial Volt/Ohm Meter with a Chopstick Electrode Set (World Precision Instruments, Sarasota, FL) on day 3 of the triculture growth and every two days subsequently. For 96-well plates, Caco-2 and HT-29MTX cells at a 3:1 ratio were seeded at a total 3×10^4 cells/well (100 μl of cell mixture) in Black-wall, clear optical bottom plates (BD Biosciences, Billerica, MA). Media was changed after four days and subsequently every other day until day 17. Raji B cells were not used in cultures grown in 96-well plates, since they are suspension feeder cells that induce M-cell differentiation of Caco-2 cells, and are not and should not be part of the epithelial monolayer itself, but could potentially adhere to the mucus surface or become incorporated in the monolayer if applied above the cells. Toxicology experiments were initiated on day 17.

Exposure of triculture to digesta—The final digesta from simulated digestions were combined with DMEM media in a ratio of 1:3, and the mixture was applied to the cells (1.5 ml to the apical compartment for transwell inserts, 200 μl per well for 96-well plates). Apical fluid in untreated control wells was replaced with fresh media. Digesta was also dispensed in a cell-free control well. Cells were incubated with digesta for 24 h. At the end of exposure TEER in transwells was measured as described earlier. Supernatant from transwells was collected for LDH analysis. Analysis of ROS production was performed after 6 h exposure using cells in 96-well plates.

Cytotoxicity assessment—Release of LDH was measured using the Pierce LDH assay kit (Sigma Aldrich, St. Louis, MO) according to manufacturer's instructions. Untreated control wells were used to measure spontaneous LDH release. For maximum LDH release control wells 150 μl of apical fluid was removed and replaced with 150 μl 10X lysis buffer 45 minutes prior to the end of incubation. The provided vial of substrate was dissolved in 11.4 ml of ultrapure water and added to 0.6 ml assay buffer to prepare the reaction mixture. Apical fluid in each well was gently pipetted to mix and 150 μl was transferred to a 1.5 ml tube. Tubes were centrifuged at 5,000 × g for 5 min., and 50 μl of the supernatant from each tube was dispensed in triplicate wells in a fresh 96-well plate. 50 μl of reaction mixture was added and mixed by tapping the plate. Plates were incubated at room temperature for 30 minutes or less (determined by visual inspection to provide maximum difference in color between samples), and 50 ml stop solution was added and mixed by tapping. Absorbance was measured at 490 nm (A_{490}) and 680 nm (A_{680}). To calculate LDH activity, A_{680} values were subtracted from corresponding measured A_{490} values to correct for instrument background signal. To correct for background from digesta, LDH activity from cell-free controls were subtracted from corresponding test well LDH activity values. Percent cytotoxicity was calculated, as recommended by the manufacturer of the assay kit, by subtracting spontaneous LDH release control values from treatment LDH values, dividing the result by the total LDH activity [(Maximum LDH release control activity) – Spontaneous LDH Release control activity)], and multiplying by 100.

ROS production assessment—Production of ROS (oxidative stress) was assessed in cells grown and treated in 96-well plates using the CellROX® green reagent (Thermo Fisher, Waltham MA) according to manufacturer's instructions. Briefly, a 5 mM working solution of CellROX® green was prepared from 20 mM stock by diluting in DMEM media without FBS. Media was removed from test wells and replaced with 100 μl working solution. Plates were incubated at 37 °C for 30 minutes. Wells were washed 3 times with 200 μl PBS, and fluorescence was measured at 480 nm (excitation)/520 nm (emission).

Acute in vivo evaluation of toxicity using a rat model.

All animal experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 8th edition, 2011), and were approved by the Harvard Medical Area Institutional Animal Care and Use Committee (IACUC), as required by the U.S. National Institutes of Health Public Health Service Policy on Humane Care and Use of Laboratory Animals. Male Wistar Han rats (12 weeks old) were obtained from Charles River Laboratories (Wilmington, MA) and were housed in standard micro isolator cages under controlled conditions of temperature, humidity, and light at the Harvard Center for Comparative Medicine. They were fed commercial chow (PicoLab Rodent Diet 5053, Framingham, MA) and were provided with reverse-osmosis purified water *ad libitum*. The animals were acclimatized in the facility for 7 days before the start of experiments.

Rats were fasted for 24 hours before each gavage and blood collection. Each rat was anesthetized with 3–4% vaporized isoflurane (Piramal Healthcare, Bethlehem, PA) and weighed. Either water, cream (20% fat), 1% CNF-50, or cream + CNF-50 (n=13 rats/group) test suspension was delivered to the stomach via the esophagus using a 4-inch, 19G gavage

needle with 2.25 mm ball tip. The volume dose was 10 ml/kg. Animals were returned to cages and were awake for the remainder of the experiment. Venous blood samples (100 μl) were collected via the tail vein prior to the first gavage. Gavage was repeated twice per week for 5 weeks. At the end of the experiment (24 h after final gavage) the animals were anesthetized, and as much blood as possible was collected via the abdominal aorta. The small intestine, liver, lung, spleen and kidney were harvested for histological analysis. A portion of each blood sample was allowed to clot and the serum was separated by centrifugation at 5000 × g for 10 minutes. Analysis of whole blood and serum markers was performed by Charles River Laboratories (Wilmington, MA).

Statistical analysis

Statistical analysis of data was performed using Prism 7.03 software (GraphPad Software, Inc., San Diego, CA). Results of TEER, *in vitro* cytotoxicity and ROS generation, as well as blood counts, hematological parameters and serum markers were analyzed by one-way ANOVA with Dunnett's multiple comparisons test.

Results

Physico-chemical and morphological properties of nanomaterials

Materials used in this study included fibrillar nanocellulose with a mean diameter of approximately 64 nm, (CNF-50), fibrillar micron-scale cellulose (MC) with mean diameter of approximately 27 μm, cellulose nanocrystals with a mean crystal diameter of approximately 25 nm (CNC-25 nm), and food grade TiO_2 (E171). Characterization data for the materials and are summarized in Table 1, and representative electron micrographs are shown in Figure 2. All materials were free of endotoxin (below limit of detection for assay, 0.5 EU/mg) and were free of bacterial and fungal contamination.

In vitro toxicity

In vitro cellular toxicity was evaluated for cellulose nanofibrils (CNF-50) and cellulose nanocrystals (CNC-25) as outlined above and compared to micron-scale cellulose (MC) (the corresponding GRAS material), as well as the commonly-used GRAS metal oxide material TiO_2 (E171). Results of these studies are shown in Figure 3.

Figure 3a shows average changes in TEER in transwells during growth and maturation of the monolayer. The TEER value increased from <100 $\Omega \cdot cm^2$ at 3 days incubation to ~2500 $\Omega \cdot cm^2$ at 17 days (immediately prior to treatment). Figure 3b shows TEER values after 24 hours of treatment with digesta from nanomaterials in fasting food model (5 mM phosphate buffer) compared to TEER in control wells (treated with digesta from fasting diet alone). No differences in TEER were seen between treatments and control, indicating that cell layer integrity remained intact. Figure 3c shows % cytotoxicity from LDH assay (% of maximum LDH release measured in lysis controls) after 24 h of exposure to intestinal digesta from fasting diet alone (control) or containing test materials at concentrations of 0.75 and 1.5% w/w. A slight (~12%) but statistically insignificant increase in cytotoxicity was observed for CNC at a starting (in food model) concentration of 1.5% w/w. Similarly small, but statistically insignificant, increases in % cytotoxicity were observed for MC at both 0.75 and

1.5% w/w and 0.75% w/w TiO_2, and a 20%, statistically significant ($p<0.01$) increase was seen with 1.5% w/w TiO_2.

Figure 3 d shows ROS production for each treatment. A slight (<1.1 fold), but statistically significant ($p<0.01$) increase in ROS production was seen with CNC at 1.5% w/w. Small but statistically significant ($p<0.01$) increases in ROS production were also seen with MC and TiO_2 at both 0.75 and 1.5 % w/w.

A standardized diet based on the average American diet was used to evaluate the effect of food model on nanocellulose cytotoxicity. Because CNC was the only material to have a significant effect (on ROS but not cytotoxicity) in fasting diet, only CNC was tested. The results of these experiments are shown in Figure 3 e and f. No increases over untreated controls, either in % cytotoxicity by LDH assay or ROS production, were observed for CNC at either 0.75 or 1.5% w/w in the standardized food model, suggesting that co-ingestion of food may ameliorate any potential cytotoxicity of CNC to some extent.

In vivo toxicity assessment

In order to assess *in vivo* toxicity of CNF-50, a semi-chronic exposure design was employed in rats (Wistar Han) who were given the different formulations by gavage, as detailed in methods. CNF was selected for these studies because of its more notable and numerous potential food applications[4], relative to CNC, and particularly its potential to reduce digestion and absorbance of fat[18]. A summary of the experimental design and results of blood and serum analyses are shown in Figure 4. A moderate but statistically insignificant reduction in weight gain was observed between rats receiving CNF-50 alone and all other groups (Figure 4 b). Rats receiving CNF-50 alone gained on average 30–40% less weight than other groups during the five weeks of treatment. No substantial or significant differences were observed between groups in terms of blood counts, including total white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils and red blood cells (Figure 4 c). Likewise, there were no significant differences in hematological measurements, including hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count or mean platelet volume between CNF-50 exposed and non exposed controls (Figure 4 c). Similarly, there were no differences between groups in serum markers including lipid profile (total cholesterol, high density lipoprotein, low density lipoprotein, free fatty acid), markers of hepatic function (alanine amino transaminase, aspartate amino transaminase, alkaline phosphatase, total protein and albumin), markers of renal function (total bilirubin, creatinine) or electrolytes (sodium, potassium, chloride) (Figure 4 d).

Histopathology revealed no significant findings in the liver, spleen, kidneys or small intestine of any animals (data not shown). A few scattered nodules of macrophages containing phagocytosed foreign material and perivasculitis, were observed in the lungs of some animals who had received either food alone or food + CNF-50. These lesions were likely the result of aspiration during or shortly after gavage.

Discussion and Conclusions

We have shown using *in vitro* methods that CNF-50 and CNC-25, after simulated digestion, are associated with minimal or no cytotoxicity in a cellular model of the intestinal epithelium. Slight but statistically insignificant increases in cytotoxicity over controls (exposed to digesta without ENM) were observed in cells treated with small intestinal digesta from fasting diet containing CNC-25 at 1.5% w/w, as well as 0.75 and 1.5% w/w MC. No increase in cytotoxicity was observed with CNF-50 or with CNC-25 at 0.75 % w/w. By contrast, a statistically significant ($p<0.01$) 20% increase was seen in cells treated with digesta from fasting diet containing 1.5 % w/w TiO_2 w/w, in line with the known bioactivity of TiO_2 ENMs.

Similarly, no significant increase in ROS production was observed at either dose of CNF-50, or at 0.75 % w/w CNC-25. A small (1.1 fold, $p<0.01$) increase in ROS production was seen with CNC at 1.5% w/w. Statistically significant increases in ROS ($p<0.01$) were also seen with both MC and TiO_2 at both doses. Though relatively small, the increase in ROS production, and corresponding small, though statistically insignificant, increase in cytotoxicity observed at the higher dose of CNC-25 may be meaningful, and suggest that further study is needed. Likewise, the modest and significant increases in ROS production, and corresponding small increases in cytotoxicity, observed with MC and TiO_2 exposures suggest that these materials may not be safe. Indeed, in the case of TiO_2 this has been borne out in numerous studies[35,36,48–54], and has led France to announce its intention to ban the use of TiO_2 as a food additive. Finally, when dispersed in a standardized food model rather than phosphate buffer alone, no increase over control in either cytotoxicity or ROS production was observed with CNC-25, suggesting that the effect of a more complex food matrix in this case was to eliminate the slight toxicity observed in the fasting diet. It should be noted that the in vitro toxicological endpoints evaluated in these studies are by no means comprehensive, and that although changes in ROS production are often an early harbinger of cytotoxicity, potential effects on endpoints not examined here, such as metabolic activity, proliferation, apoptosis, and genotoxicity, have not been excluded, and further studies are warranted to assess these endpoints.

It was also shown in an acute rat gavage model, that CNF-50 ingestion, with or without food, has no significant effects on an extensive panel of whole blood and serum biomarkers. Likewise no pathological findings were observed in liver, kidney, spleen or intestine samples from these animals. Small inflammatory nodules were noted in lungs of some rats who had received food alone or food with CNF-50. These are likely due to aspiration of gastric contents during or following gavage. It should be noted that these negative findings for CNF-50 cannot be extrapolated to CNC-25, and given that CNC-25 caused a slight increase in ROS in in vitro studies, it should be evaluated in future animal studies as well.

The finding that rats receiving CNF-50 alone gained 30–40% less weight than other groups during subacute gavage studies, though not statistically significant, is worthy of consideration and further study. In previous single gavage studies by the authors in rats, as well as in in vivo digestion and cellular studies, it was shown that CNF-50 reduces digestion of fat, and blunts the postprandial rise in serum triglycerides following a high-fat (heavy

cream) meal (gavage)[18]. Interestingly, in the semiacute multiple gavage studies reported here, no differences in weight gain were observed between rats receiving gavage meals of high fat alone and those receiving high fat plus CNF-50 or those receiving only water. Chronic feeding studies and feeding studies in obese animal models may help to explain these findings, and to determine whether such an ingested CNF material could provide a useful adjunct in the prevention and treatment of obesity.

Taken together, these primarily negative findings suggest that NC is likely non-toxic or minimally toxic compared to other GRAS ENMs. Further studies are needed for both CNF and CNC materials, including chronic *in vivo* feeding studies and assessment of other potential endpoints, such as microbiome interactions and effects on nutrient absorption. Such toxicological studies are of high importance for the food nanotechnology industry and would enable the industry to take advantage of the unique properties of these materials to improve many aspects of food quality and appeal. More importantly, this would enable the development of additional new applications for these materials, including potential uses to favorably modulate the digestion and absorbance of nutrients from the GI tract. The ability of CNF to reduce fat absorption, for example, could lead to development of foods or supplements that reduce caloric and fat uptake, which in turn could provide an additional tool to combat the worldwide obesity epidemic, which is a major healthcare challenge and cost burden[55–58].

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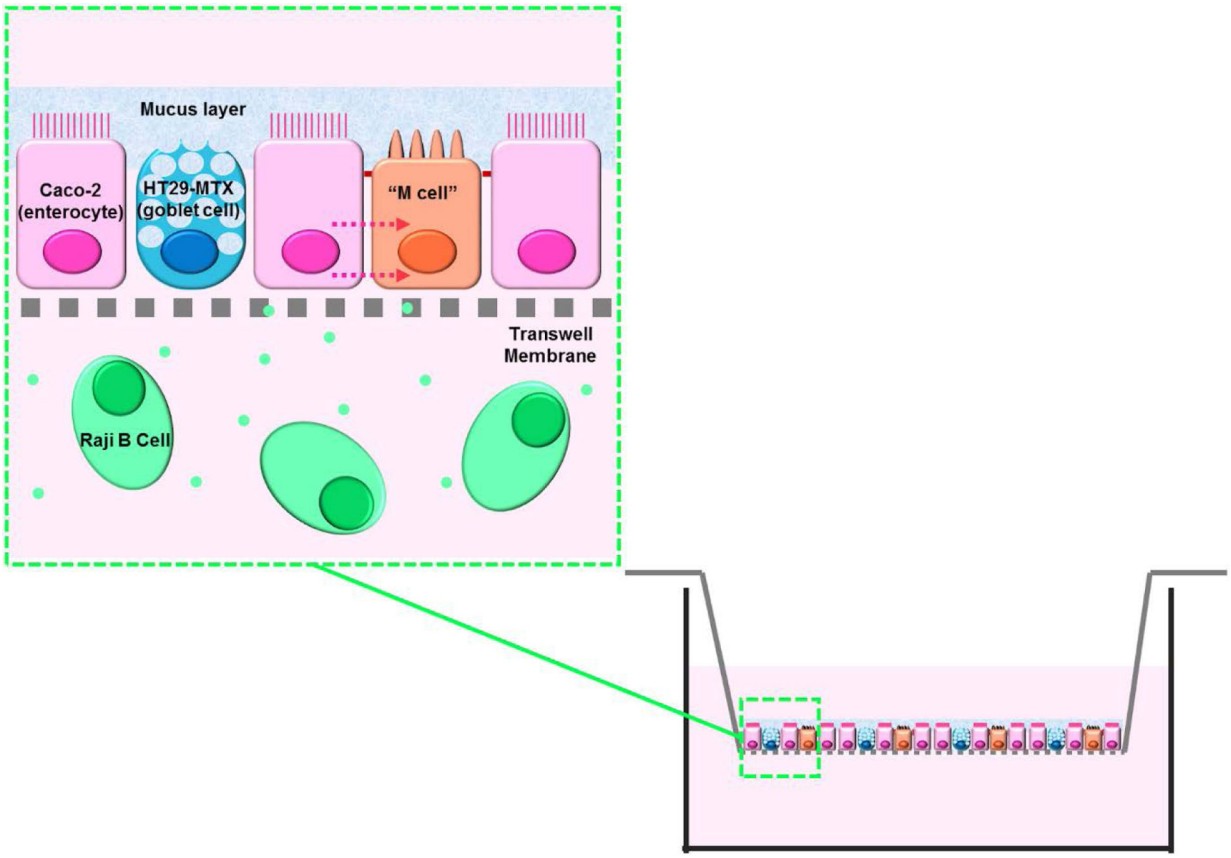

Figure 1. Triculture cell model:

Caco-2 and HT29-MTX (representing goblet cells) are co-cultured on transwell inserts for 15 days, resulting in maturation of Caco-2 to a small intestinal enterocyte phenotype. Raji B cells are added to the basolateral compartment on day 15 and the transwells are incubated an additional 2 days. Factors released by Raji B cells induce differentiation of a portion of Caco-2/enterocytes to a microfold (M) cell phenotype.

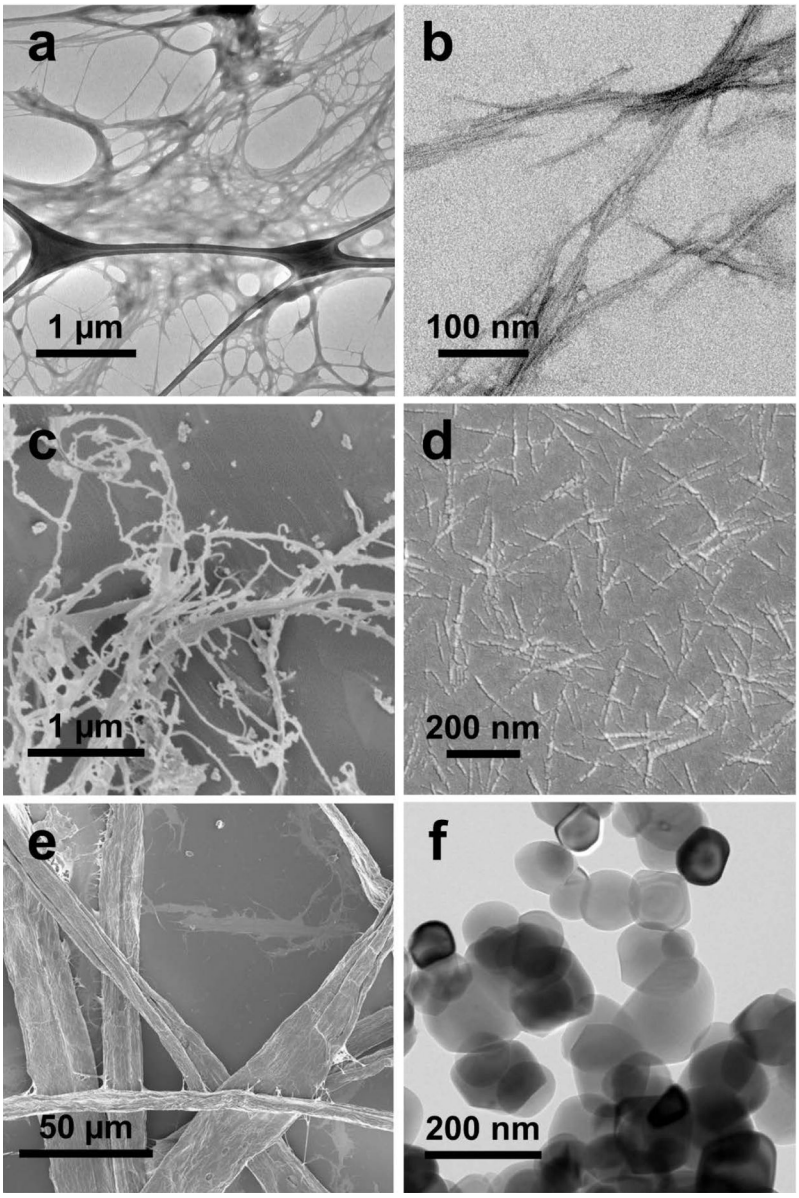

Figure 2. Electron microscopy of nanocellulose materials:

a. TEM image of 50 nm Fibrillar nanocellulose (CNF-50). **b**. TEM image of cellulose nanocrystals (CNC-25). **c**. SEM image of 50 nm Fibrillar nanocellulose (CNF-50). **d**. SEM image of cellulose nanocrystals (CNC-25). **e**. SEM image of micron-scale cellulose (MC) **f**. TEM image of TiO_2 (E171)

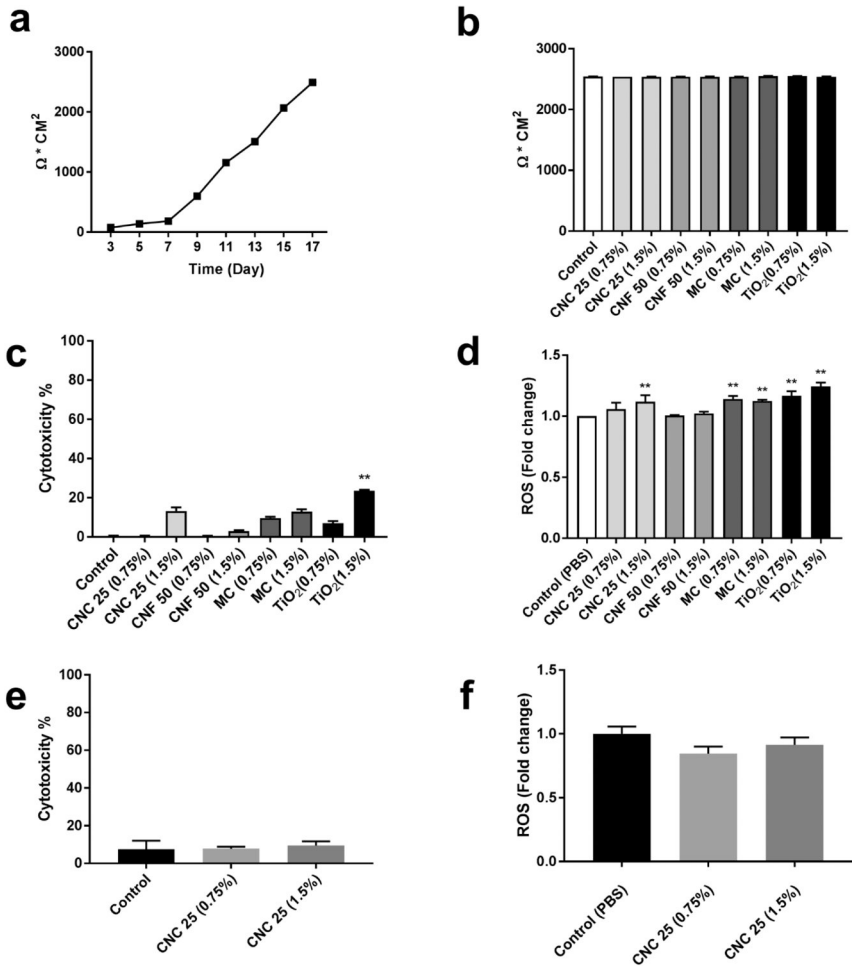

Figure 3. ***In vitro*** **toxicity:**

a. TEER measurements of triculture epithelial model in transwell inserts over time during cell growth. **b**. TEER measurements following 24 h exposures to digesta from simulated digestion of phosphate buffer (fasting food model) alone (control) or containing indicated nanomaterials. **c**. % cytotoxicity from LDH assay (% of maximum LDH release measured in lysis controls) after 24 h of exposure to intestinal digesta from fasting diet alone (control) or containing test materials at concentrations indicated. **d**. ROS generation (fold change from control) following 24 h following 24 h exposures from to digesta from fasting food model alone (control) or containing indicated nanomaterials. **e**. Cytotoxicity (LDH assay) following 24 h exposure to digesta from standardized food model alone (control) or with CNC-25 at the indicated concentrations. **f**. ROS generation following 24 h exposure to digesta from standardized food model. CNC-25: cellulose nanocrystals, CNF-50: Cellulose nanofibers, MC: micron scale cellulose fibers. All means represent results of three independent experiments with duplicate wells for each experiment. Error bars represent ±SD, *=p<0.05, **=p<.01.

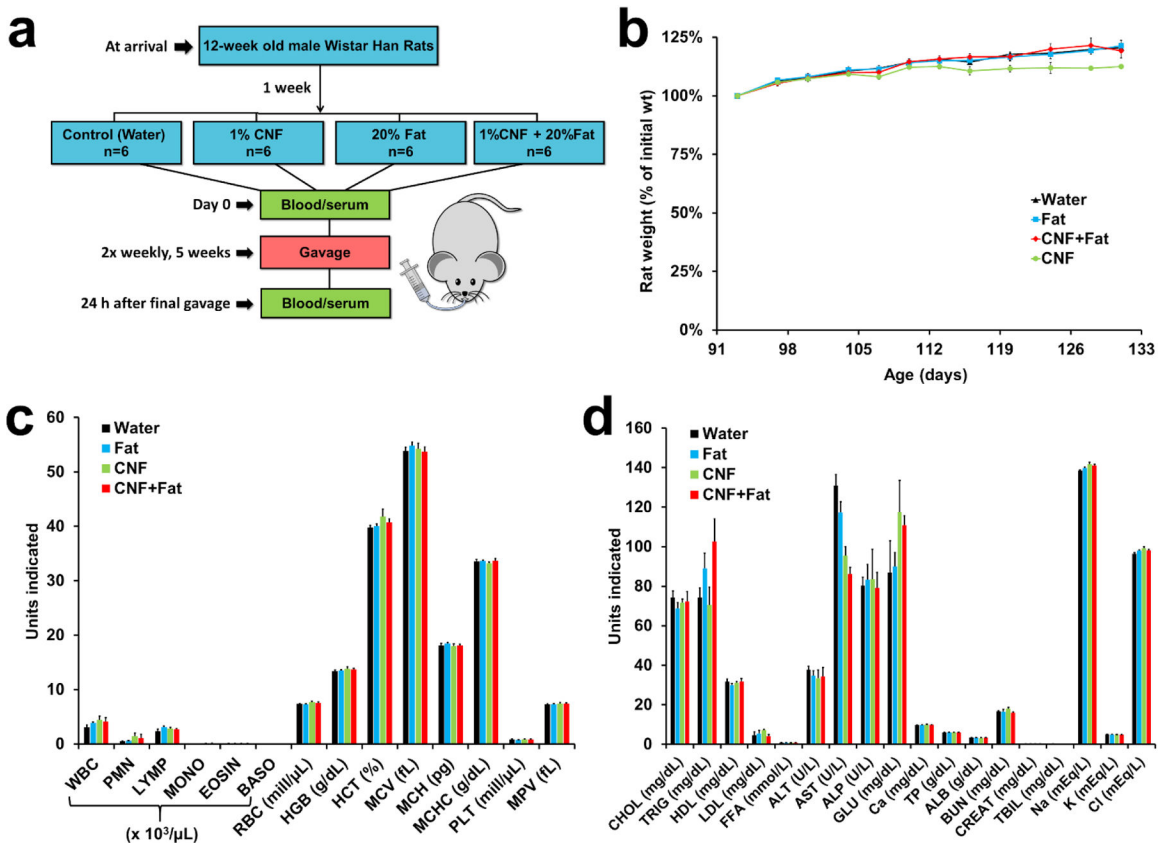

Figure 4. ***In vivo*** **toxicity in Wistar Han rats:**

a. Overview of experimental design. **b**. Mean weight of rats in each group over time. **c**. Blood differential counts and hematological parameters at end of study. **d**. Serum markers at end of study. CNF: Cellulose nanofibers (CNF-50). WBC: White Blood Cell; PMN: Neutrophil Count; LYM: Lymphocyte Count; MONO: Monocyte Count; EOSIN: Eosinophil Count; BASO: Basophil Count; RBC: Red Blood Cell Count; HGB: Hemoglobin Concentration; HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; PLT: Platelet Count; MPV: Mean Platelet Volume; CHOL: Cholesterol, Total; TRIG: Triglycerides; HDL: Cholesterol, High Density Lipoprotein; LDL: Cholesterol, Low Density Lipoprotein; FFA: Free Fatty Acid; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; ALP: Alkaline Phosphatase; GLU: Glucose; Ca: Calcium, Total; TP: Protein, Total; ALB: Albumin; BUN: Urea Nitrogen; CREAT: Creatinine; TBIL: Bilirubin, Total; Na: Sodium; K: Potassium; Cl: Chloride

Table 1:

Characterization of materials used in this study

ENMs	Physical Properties (SEM measurements)			Density (g/cm^3)	Specific surface area (SSA) (m^2/g)	Endotoxin (EU/mg)	Microbiol. Sterility (CFU/g)
	Length (μm)	Diameter (μm)	Aspect Ratio				
CNF-50	6.71 ± 5.61	0.064 ± 0.029	N/A	1.312 ± 0.016	34	< LOD *	0
CNC-25	0.27 ± 0.09	0.025 ± 0.009	11.5 ± 3.2	1.572 ± 0.021	93	1.47	0
MC	-	27.3 ± 8.8	-	-	-	<LOD *	0
	d_{XRD} (nm)	d_{BET} (nm)	d_{TEM} (nm)				
TiO_2 (E171)	55.6	103.2 ± 5.1	113.4 ± 37.2		14.44 ± 0.72	< LOD *	0

* Endotoxin Assay LOD 0.5 EU/mg (~0.05 ng/ml)