Evaluation of the genotoxic potential of different types of nanofibrillated celluloses

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ABSTRACT

Nanofibrillar cellulose (NFC) is among the most promising innovations in the forest industry. As NFC consists of thin and long fibres with a high aspect ratio, it is important to investigate the safety of NFC at an early stage of product development. Our objective was to examine the genotoxicity of four NFC materials in comparison with a bulk-sized cellulose material. *In vitro* genotoxicity was assessed in BEAS-2B cells by the comet and the cytokinesis-block micronucleus assays. *In vivo*, the comet assay was performed in lung and bronchoalveolar lavage (BAL) fluid cells and the micronucleus assay in bone marrow polychromatic erythrocytes, after single pharyngeal aspiration in C57BL/6 mice. The NFC materials tested were not genotoxic *in vitro*. All NFCs, except one, and the bulk cellulose caused DNA damage in mouse lung or BAL cells, but no systemic genotoxicity. The outcome of the *in vivo* studies was not predicted by the *in vitro* tests, suggesting that the mechanisms responsible for the *in vivo* effects were not present in the *in vitro* cell system.

Keywords: nanofibrillar cellulose, BEAS-2B cells, pharyngeal aspiration, genotoxicity

1 INTRODUCTION

Cellulose nanomaterials (CNs) derived from wood fibers are renewable and sustainable materials with wide applicability for use as bio-based composite materials, replacing fossil-based materials, in a broad range of consumer products [1]. Existing and novel applications include high quality paper, coatings, food, nanocomposite formulation and reinforcement, and biomedical applications such as drug delivery carriers, cell culture support, substitute implants, tissue repair and regeneration, and antimicrobial materials [2, 3, 4]. Because their nanoscale features may impart novel chemical properties and biological behavior, as compared with conventional cellulosic materials, it is necessary to address the safety aspects of CNs, to ensure safe working environments before scaling up their production [1].

Toxicological data on nanocellulose, especially the fibrillate form, are still very scarce, because many of the new materials are still under development. In addition, information about exposure to nanocellulose in industrial scale is still lacking. The most probable route of exposure at workplaces is via inhalation and skin [1]. NFC is a fibrous material with a high aspect ratio and this raises concern that inhaled NFC could act similarly as nano- and microsized mineral fibres. However, nanocellulose fibres are usually flexible and tangled, which might make them less harmful than long, rigid, staright nanofibres, such as certain type of MWCNTs. According to current knowledge, nanocellulose appears to have limited toxic potential [5-7]. Only a few studies on the possible genotoxicity of nanocellulose exist and most of the studies have suggested that nanocellulose is non-genotoxic [8, 9, 10, 11,].

In vivo studies on the possible toxicity of nanocellulose are also very scarce. In C57Bl/6 mice exposed to cellulose nanocrystals (CNCs) by pharyngeal aspiration, CNCs were observed to induce inflammation and impair lung functions [12]. The effects were markedly more pronounced in female than male mice, suggesting gender differences in responses to respirable CNCs [13]. To our knowledge, the only available data on the *in vivo* genotoxic and immunotoxic effects of NFC materials are those by Catalán et al [14]. In that study, a single pharyngeal aspiration of female C57BL/6 mice with TEMPO-oxidized NFC led to an acute inflammatory response and to an increased level of DNA damage in lung cells (as assessed by the comet assay) but not in cells collecetd by bronchoalveolar lavage (BAL), 24 h after the exposure. No effect was seen in the bone marrow micronucleus (MN) assay [14].

In the present study, we examined the genotoxic effects of four NFC materials both *in vitro* in BEAS-2B cells and *in vivo* in lungs of C57Bl/6 mice by single pharyngeal aspiration. The NFCs were compared to a bulk-sized pulp. To our knowledge, this is the first study where *in vitro* and *in vivo* genotoxicity outcomes have been compared for the same cellulosic materials.

2 MATERIALS AND METHODS

2.1 Materials and their characterization

Four nanofibrillar cellulose (NFC) materials (F and MC NFC, supplied by Stora Enso; AS and NS NFC, supplied by UPM Kymmene) and a bulk-sized reference material (UPM Kymmene) were used. NFCs are viscous gel-type materials

which are mainly used in wet form and they consist of long flexible and entangled fibers. The manufacturers have provided basic information on the material properties, as shown in Table 1. All cellulose samples were derived from natural wood-based pulp and consisted of glucose, xylose and mannose, hemicellulose content being 17–19 %. The materials were produced in pilot-scale factories. The same sample batches were used in all experiments.

Before toxicity assessment, all five cellulose materials were tested for possible microbial contamination by detecting possible endotoxin content, using the *Limulus Amebocyte* Lysate (LAL) method, according to the manufacturer's instructions.

Material	Conc. (%)	Length (µm)	Width (nm)	Zeta potential. (mV)
F NFC	2.40	2-20	2-15	-15
MC NFC	1.60	2-50	3-10	-32
AS NFC*	0.79	0.5-10	4-10	-25
NS NFC	1.47	2-20	7-20	-2
Bleached birch pulp	4.30	10 000	30 000	

Table 1. Properties of the cellulosic materials in their original water-based gel form.*Biocide added.

2.2 Dispersions

The NCF materials were tested as aqueous dispersions. Due to the high viscosity of the materials, stock dispersions were prepared using 1-ml syringes or by weighting the material. The materials were suspended in serum-free Bronchial Epithelial Growth Medium (BEGM) for *in vitro* experiments, and in phosphate buffered saline (PBS) for *in vivo* experiments, followed by shaking and high-speed vortexing for 10 min. The dispersions were used for the exposure immediately after preparation.

2.4 *In vitro* experiments

Transformed human bronchial epithelial BEAS-2B cells exhibiting an epithelial phenotype were used for the *in vitro* experiments. The BEAS-2B cells were grown in serum-free BEGM at 37 °C in a humified atmosphere of 5 % CO_2.

The cytotoxicity of the materials was examined in semi-confluent BEAS-2B cells on 24-well plates, to define the dose range to be used in the genotoxicity tests. Exposure times were 24 and 48 h, and a concentration range of 2.5 - 250 µg/cm^2 (corresponding to 9.5 – 950 µg/ml) was used. After the exposure, the cells were collected by trypsination, and cytotoxicity was determined by cell counts, using the Trypan Blue dye exclusion assay. The number of cells in the treated cell cultures was compared with the number of cells in the untreated control cultures.

The comet (single cell gel electrophoresis) assay was used to study DNA strand breaks and alkaline labile sites in BEAS-2B cells after the cellulose exposures. BEAS-2B cells in log phase were plated in 24-well plates two days prior to exposure. The cells were exposed for 24 h to 2.5, 5, 15, 30, 60, 120 and 250 µg/cm^2 (corresponding to 9.5, 19, 57, 114, 228, 456, and 950 µg/ml) of each cellulose material. The comet assay was performed in alkaline conditions (pH>13), as described previously [15].

The MN assay was applied to study chromosomal damage in BEAS-2B cells after exposure to the cellulose materials. 250,000 BEAS-2B cells were plated in T25 flasks three days prior to exposure. The cells were exposed for 48 h to five doses of each material in BEGM medium: 5, 20, 50, 100 and 250 µg/cm^2 (corresponding to 25, 100, 250, 500, and 1250 µg/ml). The MN assay was performed as described previously [16].

2.5 *In vivo* experiments

NFC dispersions were administered to female C57Bl/6 mice (7-8 weeks old; ~20 g per mouse) by single pharyngeal aspiration. Six mice per group were exposed to four doses of each NFC: 10, 40, 80, and 200 µg/mouse, PBS alone (negative control group), or a single 1-mg dose of tungsten carbide cobalt mixture (WC-Co; positive control group). As AS NFC contained a biocide, a separate biocide control group receiving the same biocide at a concentration corresponding to the amount present at the highest dose of the NFC was added to the test series. Cell samples were collected after a 24-h exposure, to assess acute effects, or after 28 days, for the assessment of subacute responses. The mice were sacrificed using an overdose of isoflurane, and cell samples were taken as previous described [14].

The alkaline comet assay was performed for BAL and lung cell suspensions and the MN assay in bone marrow erythrocytes as previously described [14].

3 RESULTS

3.1 Endotoxin determination

Two of the materials, MC NFC (endotoxin content 1.33 EU/ml) and F NFC (endotoxin content 1.27 EU/ml), exceeded the 0.5 EU/ml limit. In addition, the NFC materials were also tested for yeast and mold contamination. No yeast or mold colonies were detected after a 72-h incubation.

3.2 *In vitro* experiments

None of the NFCs was able to reduce cell viability below the 55±5%. limit, requested in the genotoxicity test guidelines for choosinf the top dose at, at any of the dose levels or

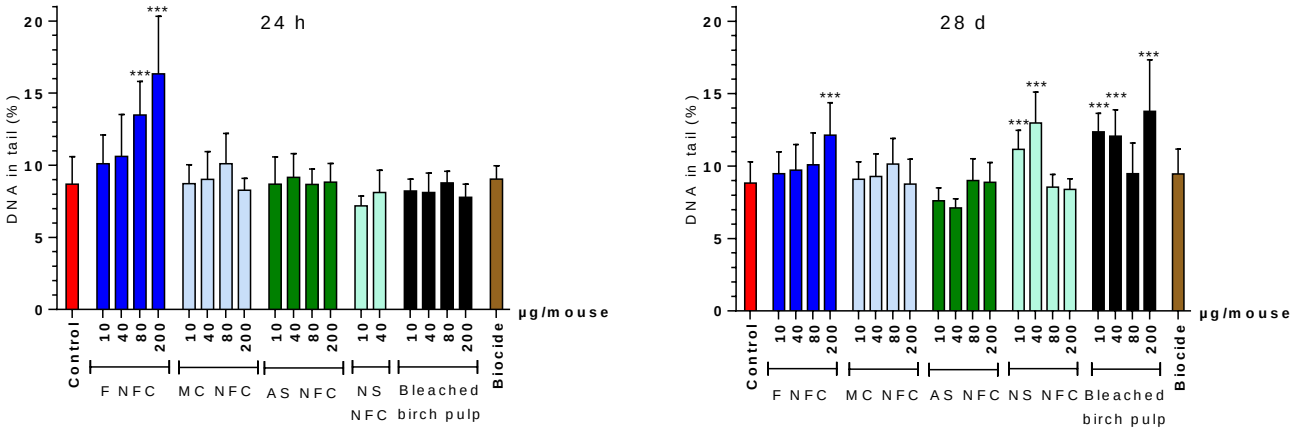

Figure 1: Mean (±SD) percentage of DNA in comet tail in lung cells after pharyngeal aspiration of the cellulose materials.

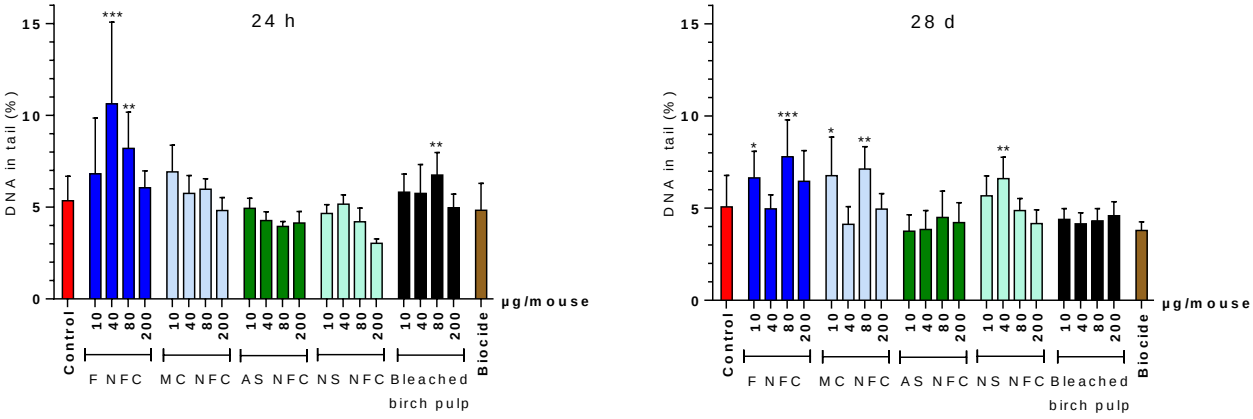

Figure 2: Mean (±SD) percentage of DNA in comet tail in BAL cells after pharyngeal aspiration of the cellulose materials.

or time points used. Therefore, the highest dose included in the genotoxicity tests was set at 250 µg/cm^2.

None of the NFC materials tested increased the level of DNA strand breaks in BEAS-2B cells as compared with the negative control after the 24-h exposure. Only for the bulk-sized reference material, bleached birch pulp, was there some indication of a possible slight induction of DNA damage at 5–30 µg/cm^2, but the effect was neither statistically significant nor dose-dependent.

No significant induction of micronuclei or decrease in cytokinesis-block proliferation index (CBPI) was seen in BEAS-2B cells after the 48-h exposure by any of the materials tested (data not shown).

3.3 In vivo experiments

As shown in Fig. 1, MC NFC or AS NFC did not increase the level of DNA strand breaks in lung cells at any of the tested doses or time points. NS NFC and bleached birch pulp neither induced DNA damage 24 h post-exposure, but both materials induced DNA damage at several of the tested doses 28 d post-exposure. However, no dose-dependency was detected. F NFC induced a clear dose-dependent increase in DNA strand breaks 24 h after the exposure, and the effect could still be observed after 28 d.

In BAL cells (Fig. 2), AS NFC did not increase the level of DNA damage. Bleached birch pulp induced DNA damage in BAL cells after 24 h but no effect was seen after 28 d. MC NFC, and NS NFC did not induce DNA damage at 24 h, but an increased level of DNA strand breaks was seen after 28 d without clear dose-dependency. F NFC induced a significant increase in the level of DNA damage 24 h and 28 d after the exposure

Only one of the tested materials, MC NFC, was able to induce a significant increase in micronucleated polychromatic erythrocytes 28 d after the exposure. As the result was

positive only at the lowest dose tested, the result was considered equivocal (data not shown).

4 CONCLUSIONS

In this study, the NFC materials and the bulk-sized birch pulp tested showed no cytotoxic or genotoxic potential *in vitro* in BEAS-2B cells at the doses and time points studied.

We also performed pharyngeal aspiration studies in C57Bl/6 mice, to detect possible acute and subacute genotoxic effects of the NFCs and the bulk material. Our results showed that the NFCs, with the exception of AS NFC, and the birch pulp were able to induce primary DNA damage in the lung or BAL cells of the mice, as measured by the comet assay. In general, both lung and BAL cells showed a low-level increase in DNA damage 24 h or 28 d after the exposure. The observed genotoxic effect may be due to an indirect or secondary influence of the presence of cellulose materials in the lungs, which is still operating 28 d after the exposure. However, the variation in the level of DNA damage between individual animals was quite large. The clearest effect was seen with F NFC which induced a dose-dependent response in lung cells both 24 h and 28 d post exposure. F NFC induced DNA damage also in BAL cells at both time points, but without dose-dependency. The underlying mechanism explaining the higher genotoxic potential of F NFC (and the non-genotoxicity of AS NFC) is not presently known. The role of microbial contamination cannot completely be ruled out, but endotoxin content, as such, does not appear to explain our findings. The presence of endotoxins is expected to elicit inflammatory responses, but it is inadequately understood which features of inflammation could be associated with secondary genotoxicity.

Our results suggest that despite of an increased level of local DNA damage in the lungs, prolonged at least for 28 days post exposure, the NFC materials and the birch pulp are not able to induce systemic genotoxic effects after exposure by pharyngeal aspiration. The 28-d follow-up time is probably too short for the cellulose materials to sufficiently reach the blood circulation.

Due to the general biopersistent nature of NFCs in the lung [17], our results raise some concern, as an increase in the level of primary DNA damage could still be detected at the 28-d time point. Most of the primary DNA damage will be repaired, but a small portion is expected to be misrepaired or to induce DNA replication errors, leading to mutational changes. If such alterations are continuously produced during a prolonged time, they might contribute to carcinogenesis. Therefore, a longer follow-up would better define the fate of the NFC material in the lungs and the duration of the increased level of DNA damage. Such studies should also include bulk-sized cellulose, as DNA damage was associated with bleached birch pulp as well. It is notable that the i*n vivo* genotoxicity of the celluloses was not predicted by the outcome of the *in vitro* genotoxicity tests, suggesting that the mechanisms responsible for the effects observed *in vivo* are not adequately present in the *in vitro* cell systems used.

FUNDING

This work was supported by UPM Kymmene Oyj, Stora Enso Oyj, the Finnish Safety and Chemicals Agency, and the European Commission, NANoREG 310584.

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