Immunotoxic and genotoxic effects after short-term inhalation of fibrous nanomaterials
Deliverable D 4.16

Introduction

Cellulose is a naturally occurring fibrous material that is generally considered safe. Cellulose pulp is exempted from the REACH regulation because it is considered to cause minimum risk, due to its intrinsic properties. Nanocellulose is one of the most promising innovations in the forest industry. The term nanocellulose generally refers to cellulosic materials having at least one dimension in the nanometre range. Nanocelluloses possess unique properties compared with the bulk material. Nanofibrillated cellulose (NFC) is a fibrous material with a higher surface area to volume ratio than bulk cellulose fibres. This raises some concern that NFC might act similarly to asbestos which is known to cause lung fibrosis and cancer. Toxicity studies on nanocellulose, especially the fibrillate form, are still very scarce.

The work described in this deliverable contributes to the hazard assessment of fibrous nanomaterials, by providing new information about the *in vivo* immunotoxicity and genotoxicity of NFC, in comparison with a bulk-sized cellulose and a well-studied fibrous nanomaterial, MWCNTs.

Description of Work

In vivo studies in mice were used to investigate both acute and subacute responses (24-h and 28-d follow up). For genotoxicity assessment *in vivo*, the comet assay was performed on lung cells and bronchoalveolar lavage (BAL) fluid cells and the micronucleus assay on bone marrow polychromatic erythrocytes, after single pharyngeal aspiration to female C57BL/6 mice (doses 10, 40, 80 and 200 µg/mouse). Inflammatory effects *in vivo* were assessed by investigating the influx of inflammatory cells in BAL, mRNA expression levels of relevant cytokines, and histopathological changes in the lungs (doses 10 and 40 µg/mouse).

Four NFC materials, included in the core selection of nanomaterials in the project, were tested. Also a sample of bulk-sized pulp and multiwalled carbon nanotubes (MWCNTs) were included in the study, to obtain comparative data on traditional cellulose and on another type of fibrous material.

These tests, together with *in vitro* testing performed with the same material under WP5, provide indication, whether the NFC materials studied are able to cause cellular damage or systemic effects. The study provides new information about the toxicity of NFC and thereby contributes to the hazard assessment of these materials.

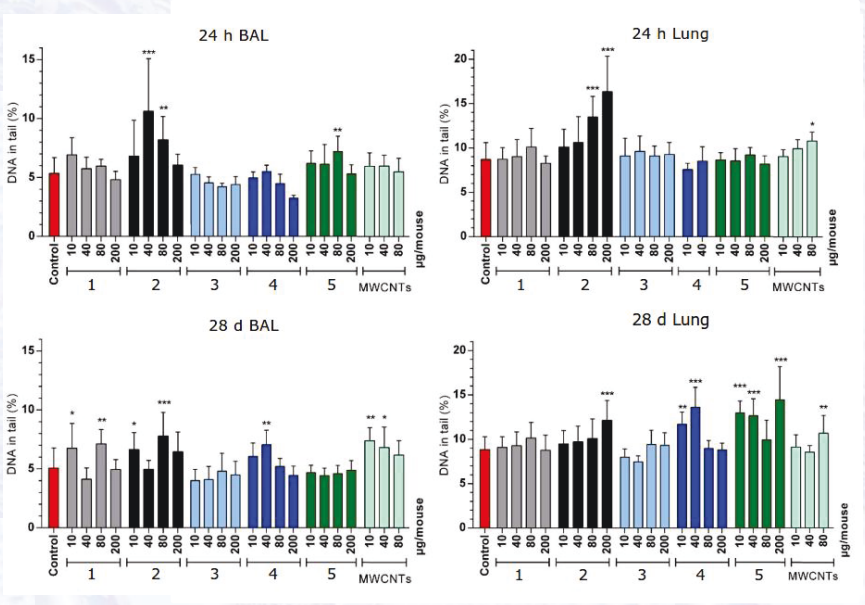
Main results and evaluation

The toxic effects observed differed among the four NFCs studied, but effects were also seen with the bulk-sized cellulose studied. As concerns inflammatory effects, the mice exposed to the NFC materials and the bulk-sized cellulose showed signs of recovery of inflammation during the 28-d study period, while the MWCNT-treated mice exhibited characteristics of longer-term adverse health effects. Based on the results of the present study, it seems that exposure through the respiratory tract to NFC can cause acute inflammatory responses, which, however, are not anymore present 28 d later.

All tested materials were able to trigger recruitment of neutrophils 24 h after the treatment. The effect was clearly stronger for two of the NFC materials. In addition, the same two NFC materials induced a slight influx of eosinophils. 28 days after the exposure, no significant increases in the numbers of neutrophils or eosinophils was seen in BAL, indicating that the inflammation had resolved.

The mRNA expression analysis of cytokines supported the results of the cytological assessment of the BAL. All materials tested were able to induce an increase in the expression of inflammation-related cytokines 24 h after the exposure. 28 d later, only a minor induction of mRNA expression was detected in response to the NFC treatment. In contrast, with MWCNTs the mRNA level of the studied cytokines still remained elevated 28 days after the exposure.

All NFC materials, except one, caused DNA damage in lung or BAL cells, as determined by the comet assay. For one NFC, the effect was dose-dependent in lung cells both 24 h and 28 days after the exposure. The comparative materials, bulk-sized pulp and MWCNTs, were also able to induce DNA damage after 24 h and 28 days. None of the NFCs or comparative materials showed systemic genotoxic properties, as measured by the micronucleus assay in bone marrow.



*In vivo comet assay (percentage of DNA in comet tail; mean ± SD). 1-4 = NFC materials, 5 = bulk-sized pulp. (***p<0.001, **p<0.01, *p<0.05; one-way ANOVA).*

Although NFCs appeared to be biopersistent during this follow-up time, they induced no pathological changes observable by histological examination of the lungs. In this sense, they markedly differed from MWCNTs which still showed clear inflammatory responses 28 d after the exposure and also produced various alterations in the histology of the lungs.

The outcome of the *in vivo* toxicity tests was not consistently predicted by the *in vitro* toxicity studies reported in WP5. None of the NFCs or the bulk-sized pulp were genotoxic *in vitro* and only one of the NFCs was able to induce inflammatory cytokines *in vitro*. This comparison suggested that the mechanisms responsible for the effects observed *in vivo* are not fully present in the *in vitro* cell systems used.

In the present study, exposure via the respiratory route was chosen to mimic a tentative worst-case-scenario, where liquid nanocellulose is aerosolized in the atmosphere during the manufacturing process and inhaled by workers. As NFC production is presently in an experimental phase, it is not yet known how realistic this kind of exposure scenario could be in the future production of NFC. In addition to inhalation of aerosols or dry fibres, possible exposure may also include dermal and oral routes.

Toxicological data on nanocelluloses are still few, which limits comparison with existing literature. Although NFC is a HARN, nanocellulose fibres are flexible and tangled. It has been suggested that this type of fibres are less harmful than long, rigid, needle-like nanofibers, such as the MWCNTs used as a reference material which in many ways resemble asbestos. Also the findings of the present study suggest that MWCNTs have more severe adverse health effects than the NFCs tested.

In conclusion, the results indicate that, although the tested NFC materials were able to induce DNA damage and inflammatory responses at 24 h, the mice exposed to the NFCs showed signs of recovery of the inflammation at 28 d. On the contrary, the mice treated with MWCNTs exhibited characteristics of longer term adverse health effects. However, the observation that the NFCs, and the bulk-sized pulp, were biopersistent in the lungs for at least 28 days raises some concern, because some increase in the level of primary DNA damage was still observed at this time point. If DNA damage is continuously produced during a prolonged time, it might contribute to carcinogenesis. A longer follow-up would be required to better define the fate of the NFC material in the lungs and the duration of the increased level of DNA damage.

For more details about NANoREG please visit the official website **www.nanoreg.eu**.

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