IN VIVO TOXICITY OF CELLULOSE NANOFIBRILLS ON MICE

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Abstract

The extensive application of nanomaterials in industry, medicine and consumer products has raised concerns about their potential toxicity. The purpose of this study was to investigate the toxicologic responses of mice to cellulose nanofibrills (CNF). The animals were randomly divided into four experimental groups: Each *group* of five mice of each sex was *administrated daily via oral gavage at dosage level of 0, 0.5, 1 and 2 g/kg b.w. CNF.* Eighteen days later, the animals were anesthetized by ether and then sacrificed. In an effort to examine spleen, liver and kidney injury, we assessed coefficients of these organs, biochemical parameters of their functions, hematological indices, and histopathological changes in mice. CNF-treated mice showed no significant differences from controls neither with respect to the biochemical parameters nor in relation to blood elements of urea, creathinin (CR), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). The uric acid (UA) level, a marker of renal function, and aspartate aminotransferase (AST) level, a hepatotoxicity indicator decreased and increased, respectively in mice dosed with CNF. These data suggested that hepatic damage occurred in treated groups. This was confirmed by histopathologic analyses which revealed that liver cell change (LLCC) and spotty necrosis of hepatocyte in the liver tissue occurred, although *no* sign of *toxicity* was observed in the spleen and *kidney* in histological examination. While there were no significant changes of the body weight coefficients of spleen and kidney, the liver coefficient increased gradually.

Keywords: Cellulose nanofirills, Toxicity, Liver

1. INTRODUCTION

Nanotechnology has been defined as using materials and structures with nanoscale dimensions, usually in the range 1–100 nm (Masciangioli and Zhang, 2003). The properties of nanoparticles (NPs) differ substantially from their respective bulk materials of the same composition. Application of NPs in electronics, optics, medical devices, drug delivery systems, chemical sensors, biosensors, and in environmental remediation, textiles, defense, agriculture, cosmetics, and other areas are already a reality and applications are beginning to impact the food associated industries (Garcia et al., 2010). In food and agricultural systems nanotechnologies cover many aspects, such as food security, packaging materials, disease treatment, delivery systems, bioavailability, new tools for molecular and cellular biology and new materials for pathogen detection (Maynard et al., 2006; Chen et al., 2006; Weiss et al., 2006). With the industrialization of the nanotechnology, public exposure to NPs should be increasing in the near future. However, certain novel properties of NPs could lead to adverse biological effects, with the potential to create toxicity (Farhadian et al. 2012); Thus, the studies on safety and (eco) toxicity of NPs are of extreme importance (Kahru et al., 2008).

Cellulose, the building material of long fibrous cells, is a highly strong natural polymer. The use of nanocellulose as a reinforcing phase in nanocomposites is a relatively new area of interest and has numerous well-known advantages e.g., low cost of the raw material, low density; renewable nature; wide

variety of filler available through the world; low energy consumption; high specific properties; biodegradability; relatively reactive surface, which can be used for grafting specific groups and almost unlimited availability [Gandini and Belgacem 2008a,b; Rafieian and Simonsen 2014, Rafieian et al. 2014]. Basically two types of nanoreinforcements can be obtained from cellulose – Microfibrillated cellulose (MFC), and whiskers. In plants or animals (tunicates) the cellulose chains are synthesized to form MFC (also known as nanocellulose or nanofibrillated cellulose) which are bundles of molecules that are elongated and stabilized by hydrogen bonds. The MFC have nanosized diameters and lengths in the micrometer range. Each microfibril is formed by aggregation of elementary fibrils, which are made up of amorphous and crystalline parts. The crystalline parts, which can be isolated by several treatments, are the nanocrystals, also known as whiskers, nanorods, or rodlike cellulose microcrystals (de Azeredo 2009). Each microfibril can be considered a string of whiskers, linked along it by amorphous domains (which act as structural defects).

Kovacs et al. (2010) investigated ecotoxicological characterization of nanocrystalline cellulose (NCC). This involved toxicity tests with rainbow trout hepatocytes and nine aquatic species. The hepatocytes were most sensitive (EC_{20}s between 10 and 200 mg/l) to NCC, although neither NCC nor carboxy methyl cellulose (CMC) caused genotoxicity. In tests with the nine species, NCC affected the reproduction of the fathead minnow at (IC25) 0.29 g/l, but no other effects on endpoints such as survival and growth occurred in the other species at concentrations below 1 g/l, which was comparable to CMC. Based on this ecotoxicological characterization, NCC was found to have low toxicity potential and environmental risk. In the present study, the mice were exposed to cellulose nanofibrills (CNF) by oral gavage and the nanotoxicity in vivo was investigated.

2. MATERIALS AND METHODS

2.1. CNF preparation

The CNF were produced by passing the water slurry with 1 wt% canola straw fibers once through a disc grinder (MKCA6–3; Masuko Sangyo, Kawaguchi, Japan) at 1500 rpm.

2.2 CNF characterization

The aqueous suspension of CNF was diluted 1:10 with methanol. One drop of diluted suspension was deposited onto a silicon wafer, followed by vacuum- oven drying at 50 °C. The morphology and size distribution of CNF dimension were analyzed by SII Nanonavi E-sweep AFM (SII Nanotechnology, Inc., Japan) worked in a dynamic force mode at 25 °C and a relative humidity (RH) of 45-55%. The Si probe was an SI-DF20 (SII Nanotechnology, Inc., Japan) with a nominal spring constant of 18 N/m and a resonance frequency of 138 kHz.

2.3. Animals and treatment

ICR mice of either sex (provided by), aged 4 weeks and weighing ~30 g, were used in the experiments. The animals of the same sex were housed in stainless steel cages containing sterile paddy husk as bedding in ventilated animal rooms. They were acclimated in the controlled environment (temperature: 20 ± 2 °C; RH: 50–70% and light: 12 h light/dark cycle) with free access to a commercial laboratory complete food and water. After 1 week, the mice were randomly divided into four groups. Each group had 5 male and 5 female mice. The animals were kept fasting over night before treatment. The groups were given by CNF at a dose of 0, 0.5, 1 and 2 g/kg body weight. After administration, the skin and fur changes, eye secretion, respiration and behavior patterns of the mice were observed daily for total 18 days. Special attention was given to the clinical signs of toxicity including tremors, convulsions, salivation, nausea, vomiting, diarrhoea, lethargy, sleep and coma. The body weight of mice was recorded before and every 3 days after the administration. 18 days later, the animals were sacrificed. The blood was obtained from ophthalmic veins. The spleen, liver, and kidney were collected and were kept in 10% formalin for histopathological examination.

2.4. Blood-element test

0.1 mL of 15 g/L EDTA-Na was added into 1 mL whole blood and the anticoagulant blood sample was immediately analyzed for the blood element within 2 h. The blood-element: white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and blood platelet (PLT) were determined by an automatic hematology analyzer (SYSMEX Co. Ltd kx-21N, Japan).

2.5. Biochemical assay of serum

The serum was obtained by centrifugation of the whole blood at 3000 rpm for 15 min. Uric acid (UA), creatinine (CR), urea, aspartate aminotransferase (AST), alkaline phosphatase (ALP) and alanine aminotransferas (ALT) levels were estimated in the serum with automated biochemical analyzer using the kits (Beckmann).

2.6. Coefficients of liver, spleen, and kidneys

After weighing the tissues and body, the coefficients of spleen, liver and kidneys, to body weight were calculated as the ratio of tissues (wet weight, mg) to body weight (g).

2.7. Histopathological observation

For conventional histology, a small piece of spleen, liver, and kidneys were taken immediately after the sacrifice of the animals, fixed in 10% formaldehyde, embedded in paraffin, sectioned for 5–6-mm thick, stained with hematoxylin and eosin and examined by light microscopy.

2.8. Statistical analysis

Results were expressed as mean ± standard deviation (SD). The Student's t-test was used to determine the significance of differences between means. A value of $P < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1. CNF characterization

AFM Analysis, presents images with near-atomic- or atomic- resolution surface topography, and is able to quantify surface roughness of samples down to the angstrom-scale. Furthermore, it can provide quantitative information about feature sizes, such as step heights and other dimensions. In this study, AFM observation (Figure 1) showed that the dispersions consisted of mostly individualized CNF with diameter of 28 ± 10 nm.

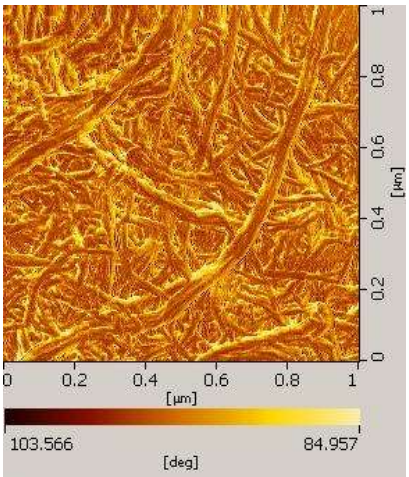

Figure 1 AFM image of dried suspension of CNF.

3.2. Blood-element test

Results of hematological study, presented in Table 1, showed no statistically significant difference in the mean levels of hematological parameters including WBC, RBC, HGB, HCT, MCV, MCH, MCHC and PLT among the four group mice. The high variance within groups meant the difference in means was not statistically significant ($P > 0.05$). We can conclude no hematological disorder in mice was induced following oral gavage of different concentrations of CNF.

Table 1 Effect of CNF (0, 0.5, 1 and 2 g/kg b.w.) on hematological blood parameters of mice after 18 consecutive days of oral administration

	(Average ± SD)			
Parameters \ Dose (g/kg)	0	0.5	1	2
WBC (/µl)	9150 ± 3425.64[a]	8180 ± 2431.19[a]	8700 ± 3778.89[a]	8900 ± 2607.20[a]
RBC (/µl)	9073333.33 ± 669975.1[a]	9509000 ± 489840.1[a]	8464000 ± 1520542[a]	9076000 ± 1268658[a]
HGB (g/dl)	12.37 ±1.32[a]	13.37 ± 0.64[a]	11.93 ± 2.06[a]	12.52 ± 2.08[a]
HCT (%)	43.37 ±3.82[a]	45.92 ± 1.97[a]	41.5 ± 6.99[a]	43.52 ± 5.83[a]
MCV (fL)	47.75 ±1.30[a]	48.35 ± 1.70[a]	49.31 ± 3.06[a]	48.03 ± 1.88[a]
MCH (pg)	13.62 ± 0.80[a]	14.08 ± 0.78[a]	14.13 ± 0.56[a]	13.75 ± 0.99[a]
MCHC (g/dl)	28.5 ± 1.31[a]	29.12 ± 0.98[a]	28.74 ± 2.26[a]	28.65 ± 1.40[a]
PLT (/µl)	1155666.67 ± 431354.9[a]	1019300 ± 337667.8[a]	963600 ± 206423.6[a]	1079500 ± 300280.7[a]

Means of three replicates with standard deviations. Any two means in the same row followed by the same letter are not significantly ($P > 0.05$) different according to Duncan's multiple range tests.

3.3. Biochemical parameters in serum

Renal dysfunction can be studied by the measurement of biochemical parameters in blood; hence we carried out this study to examine the status of UA, CR and blood urea as the possible markers for the renal functions. This clinical investigation is important especially in a setting where facilities for the definitive elaborate renal assessments are not feasible. The levels of UA were determined since hypouricemia (decrease of uric acid in the blood) is a common sign of drug toxicity.

Table 2 Hematological parameters in mice by oral administration with CNF for consecutive 18 days.

	(Average ± SD)			
Parameters \ Dose (g /kg)	0	0.5	1	2
UA (mg/dl)	6.4 ± 1.18[a]	5.76±2.08[ab]	4.46 ± 1.63[b]	4.05 ± 2.14[b]
CR (mg/dl)	0.44 ± 0.08[a]	0.47 ± 0.07[a]	0.48 ± 0.08[a]	0.47 ± 0.12[a]
Urea (mg/dl)	51.43 ± 11[a]	58.11±7.64[a]	57 ± 8.18[a]	50.7 ± 9.04[a]
AST (U/L)	134.8 ± 39.10[ab]	137.83 ± 21.83[a]	171.89 ± 35.88[b]	185.88 ± 83.99[ab]
ALP (U/L)	484.71 ± 677.19[a]	167.11 ± 103.63[a]	228.8 ± 91.41[a]	206.3 ± 72.73[a]
ALT (U/L)	60.14 ± 18.40[a]	71.8 ± 17.85[a]	67.56 ± 29.07[a]	73.67 ± 39.75[a]

Means of three replicates with standard deviations. Any two means in the same row followed by the same letter are not significantly ($P > 0.05$) different according to Duncan's multiple range tests.

According to Table 2, with high dose of CNF (1 and 2 g/kg b.w.), UA deceased but CR and blood urea did not change significantly. Also we evaluated the liver function by measuring the liver enzymes AST, ALP and ALT. There were not significant changes for the enzymes of ALP and ALT after oral administration of

different CNF concentrations ($p > 0.05$). The mice showed significantly higher level of AST at an oral dose of 1 mg/kg b.w. per day administered for consecutive 18 days compared to the mice *orally administered* 0.5 g/kg. The raised serum level of AST was ascribed to the injured healthy structure of the liver tissue, because all of the above-mentioned enzymes are cytoplasmic in their position, and are released into blood circulation after cellular injury and high levels of each factor may imply a failure of active pathologies (Salimi et al. 2014).

3.4. Coefficients of spleen, liver and kidneys

After 18 days, the mice were sacrificed and weighted; No significant differences were found in the body weight of the experimental groups. Various organs were collected and also weighted. Table 3 shows the coefficients of spleen, liver, and kidneys to body weight. There were no significant changes in the coefficients of spleen and kidneys. But the coefficient of liver in the 0.5 g/kg b.w. group was significantly higher ($p < 0.05$) than the control. The increased coefficient indicates that the inflammation might be induced in this group after ingestion of CNF, which was confirmed by the further examination of liver.

Table 3 coefficients of spleen, liver and kidney.

Tissue \ Dose (mg/g)	(Average ± SD)			
	0	0.5	1	2
Spleen	8.76±1.75^{a}	8.84±3.60^{a}	9.84±3.45^{a}	10.49±5.97^{a}
Liver	50.88 ±4.96^{a}	56.51±4.53^{b}	52.90±6.82^{ab}	53.86±10.08^{ab}
Kidney	14.70±2.45^{a}	13.62±3.77^{a}	14.30±3.21^{a}	13.52±3.18^{a}

Means of three replicates with standard deviations. Any two means in the same row followed by the same letter are not significantly ($P > 0.05$) different according to Duncan's multiple range tests.

3.5. Histopathological evaluation

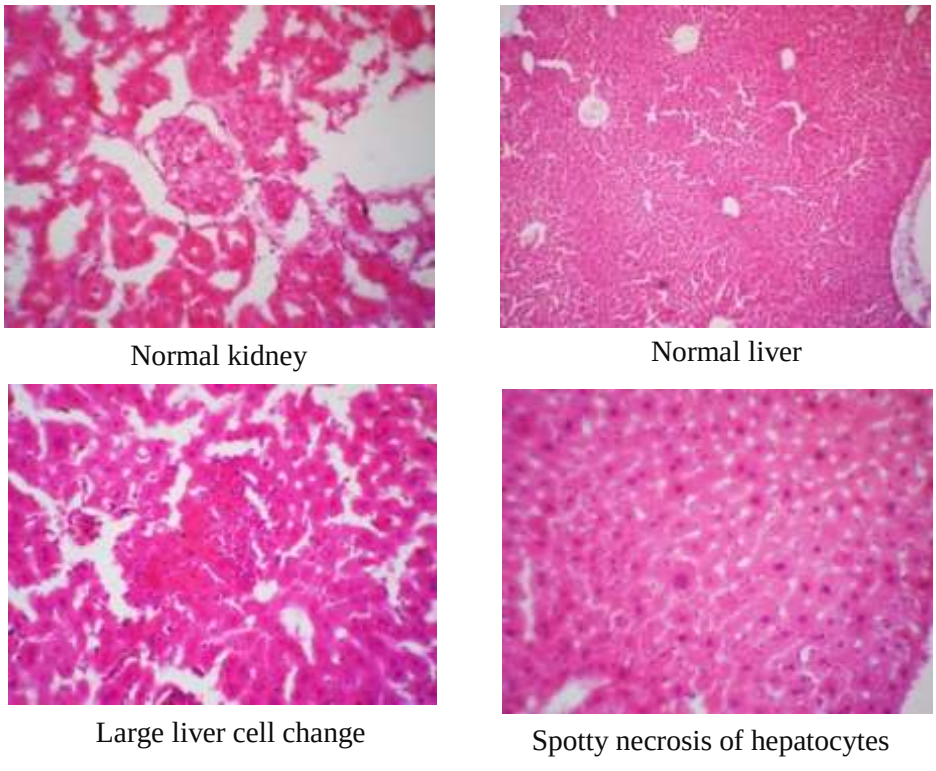

Figure 2 Histopathology of the kidney and liver tissues in mice after oral administration with various doses of CNF suspensions for consecutive 18 days.

Histopatolocal staining with hematoxylin and eosin showed the normal spleen (data not shown here) and kidney structure (Figure 2) between all groups and there was no significant damage to the glomeruli such as (hypertrophy, hypercellularity) and tubules (dilution, atrophy). Furthermore, no significant histopathological change in liver tissues of control group and mice exposed to the 0.5 g/kg was detected. We observed the large liver cell change (LLCC) and spotty necrosis of hepatocyte in the liver tissue of mice post-exposure 18 days to the 1 and 2 g/kg CNF. LLCC, also called liver cell dysplasia of large-cell type, refers to microscopic lesions (Kim et al. 2009) and is a set of cytologic changes comprising nuclear and cytoplasmic enlargement, nuclear pleomorphism, and multinucleation (Chan and Burt 2011). This entity is encountered frequently on histologic or cytologic examination of specimens obtained from livers with a variety of chronic diseases and originally was thought to have a premalignant nature. Spotty necrosis of hepatocytes indicated that the hepatic injury was induced by exposure to high concentration of CNF (Wang et al. 2006).

CONCLUSION

While nanotechnology is a rapidly expanding discipline of material sciences that is predicted to have many potential benefits, available studies indicate that nanoparticles may also have a significant potential to induce adverse outcomes. Exposure to nanoparticles is probably associated with a range of acute and chronic effects (Bakand et al. 2012). In this study, mice showed higher coefficients of liver in the treated groups with 0.5 g/kg than the control group. Alteration of AST, one of the serum biochemical parameter may be attributed to liver dysfunction. Although AST is elevated in liver disease, the elevation can also be secondary to enzyme induction without hepatic pathology (Hussein et al. 2013); so further research was needed before a conclusion is made. In the histopathological examination the hepatic injury (large liver cell change and spotty necrosis of hepatocyte) was proved.

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