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Original article

Cellulose nanocrystals induce a dose-dependent effect on cytotoxicity and proliferative activity of human peripheral blood mononuclear cells

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Summary

Introduction. Cellulose nanocrystals (CNCs) as attractive natural materials, have numerous applications in the biomedical field. Their unique biomechanical characteristics, surface chemistry, low cost and sustainable nature make them an engaging alternative to conventional materials and potentiate their use as progressive material. Therefore, it is critical to evaluate the cytocompatibility and immunomodulatory properties of nCNCs, which have not been completely explored. The objective of this study was to examine a dose-dependent effect of native (n)CNCs on cytotoxicity and proliferative activity of human peripheral blood mononuclear cells (PBMNCs) in vitro.

Methods. PBMNCs, obtained from the healthy blood donors, were cultivated with nCNCs. Cell viability was analyzed by flow cytometry assay, while proliferative activity was determined by MTT, [3H]-thymidine uptake assay and detection of IL-2 production.

Results. The cytotoxicity results suggested that no concentration of nCNCs (50-400 μg/ml) affected necrosis of PBMNCs, whereas apoptosis was induced by the highest concentration of nCNCs compared to control ($p<0.05$). Unexpectedly, the highest concentration of nCNCs increased the metabolic activity of PHA-stimulated cells compared to control ($p<0.05$). In contrast to these findings, lower concentrations of nCNCs (50 μg/ml and 100 μg/ml) stimulated proliferation of PBMNCs ($p<0.05$ and $p<0.001$). It was followed by increased production of IL-2 (100 μg/ml) ($p<0.001$).

Conclusion. The results suggest that non-cytotoxic concentrations of nCNCs modulate the proliferative activity of human PBMNCs, a phenomenon which has not been published up to now and which is relevant for further studies.

Keywords: cellulose nanocrystals, peripheral blood mononuclear cells, culture, cytotoxicity, proliferative activity

Introduction

In recent years, natural polymers, especially cellulose, present popular area of intense research in biomedicine [1]. Nanoscaled form of cellulose, (nanocellulose) attracts more attention as unique and sophisticated material. Unlike traditional materials, nanocellulose is characterized by biocompatibility, non-toxicity, biodegradability, combined with outstanding mechanical and chemical properties, low cost, availability, and sustainability [2]. Such qualities offer numerous application opportunities

of nanocellulose in various technical, nutritional, pharmaceutical and biomedical areas [3, 4].

Depending on the source and the chemical treatment, nanocellulose can be classified into three major types: cellulose nanofibrils (CNFs), cellulose nanocrystals (CNCs) and bacterial cellulose (BC). Takng into accound that cytotoxicity is a crucial parameter of biocompatibility, CNFs can be recognized as safe and biocompatible material, as pointed out by several papers [5-7]. In vitro reports concerning the CNF immunomodulatory abilities in two models of human immune cells, Čolić et al. confirmed tolerogenic capacity of CNFs [8, 9]. This was estimated by means of the inhibitory effect of CNFs on T helper1 (Th1), Th17 responses and T-cell activation, as well as by expanding Th2 cells, activating regulatory T cells (Tregs) and increasing the production of immunoregulatory cytokines. The latter study showed that phosphonation of CNFs enhanced these properties [10].

Cellulose chains parallely packed into filamentous structures formed needle-like CNCs (high-aspect-ratio nanoparticles, HARN ≥ 3). Due to the uniaxial orientation of these chains and the high degree of crystallinity, CNCs possesed tremendous stiffness and strength [11]. Having these characteristics, cellulose nanocrystals have obtained increasing attention recently as a result of the renewable nature of the source, comparatively low cost, low density, as well as its biodegradability. Other attractive features are the ability of CNCs to interact with other polymeric matrix materials and possibility of chemical modifications due to the abundance of surface hydroxyl groups [12]. All these features make CNCs attractive for a wide spectrum of new applications.

Taking into account that nanocellulose, including CNCs, has had commercial applications, controversy concerning its biocompatibility and immunomodulatory properties has been actualized. As it has been already mentioned, cellulose is generally considered to be biocompatible and safe material [2, 13, 14]. In contrast, cytotoxic and inflammatory effect of CNCs was confirmed in vivo, using a mice model [15]. Namely, pulmonary exposition to two types of CNCs such as powdered CNC (CNCP) and CNC suspension (CNCS) induced increased production of pro-inflammatory cytokines, particularly tumor necrosis factor (TNF)-α and interleukin (IL)-1α and activation of Th1 (CNCS) and Th2 (CNCP) immune responses. These results were in accordance with the results of Clift et al., who showed slight cytotoxic and inflammatory effect of CNCs in a model of human epithelial triple-cell co-culture [16]. However, such a phenomenon was not demonstrated against nine different cell lines, although applied concentrations of CNCs were similar [17]. In a realistic and efficient 3D model of the human epithelial tissue cells, Endes and co-workers tested aerosolised CNCs at a concentration of 0.14 to 1.57 μg/cm^2. After 24-hour post-exposure they did not identify cytotoxicity, oxidative stress and (pro-)inflammatory response considering the production of IL-8 and TNF-α [18]. In line with these results, Catalan and his group showed that CNCs (30–300 mg/ml) did not up-regulate the production of pro-inflammatory cytokines (IL-1β and TNF-α) in human monocyte-derived macrophages after a 6-hour exposure [19].

It's obvious that besides the biocompatibility, the immunomodulatory properties of biomaterials should be examined before the clinical applications. The interaction of nanomaterials with the components of the immune system is particulary important in a view of its protective role. To this date, despite the biocompatibility of nanocellulose, almost nothing has been known about immunological properties of native (n)CNCs. Therefore, the study objective was to investigate the effect of nCNCs on cytotoxicity, proliferation and IL-2 production of human peripheral blood mononuclear cells (PBMNCs) in culture.

Materials and methods

nCNCs and PBMNCs

nCNCs were produced and supplied by Betulium Ltd., Finland. The levels of concentrations used in cultivation ranged from 50 μg/ml to 400 μg/ml. nCNCs were prepared in complete Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal calf serum (FCS), 50 μM 2-mercaptoethanol (all from Sigma-Aldrich), and 100 U/ml penicillin, 20 μg/ml gentamicin, 100

mg/ml streptomycin (Galenika).

The mononuclear cells were obtained from the voluntary healthy blood donors (buffy coats) at the Department of Blood Transfusion, University Hospital in Foca, upon receiving a signed informed consent in accordance with the Declaration of Helsinki. Approval for all the experiments was obtained from the Ethics Comittee of the University of East Sarajevo, Faculty of Medicine in Foca (permission date October 24th 2018, Foca). Buffy coat was diluted with 0.02% ethylenediaminetetraacetic acid disodium salt dihydrate (NaEDTA) in 0.01 M phosphate buffered saline (PBS) in 1:2 M ratio and gently inflicted on the density gradient (Sigma-Aldrich). After centrifugation (1000xg, 20 min, 20 °C), the interphase containing PBMNCs was transferred to a new sterile conical tube. For removal of platelets, cells were centrifugated four times (130xg, 10 min, 20 °C) with 0.02% NaEDTA in PBS. Eventually, the mononuclears were resuspended in complete medium. After Trypan blue staining (1% in physiological solution), their viability and number were estimated using a light microscope (Olympus).

Necrosis and apoptosis

PBMNCs ($3x10^5$/well of 96-well plates (Sarstedt)) have been plated in complete medium with nCNCs (50 µg/ml-400 µg/ml) or without nCNCs (control) in an incubator with 37°C, 5% CO_2 and 90% humidity for 48 h. All cultures were set up in triplicates. After that, PBMNCs cultures were collected, filtrated and centrifuged (130xg, 10 min) for the purpose of removal of remaining nCNC in the supernatants.

After staining the PBMNCs with propidium iodide (PI) (Sigma-Aldrich) 20 µg/ml in PBS, the viability was determined using flow cytometry (Sysmex Partec Cube 6). Necrotic cells were determined as PI+ cells. Minimal number of analyzed cells was 5.000 within a sample. The results are shown as percentages of necrotic cells.

PI (50 µg/ml) staining of mononuclears in hypotonic citric/Triton-X buffer was used for apoptosis detection. The analysis was done by cytofluorometery, as specified in necrosis assay. Hypodiploid cells (sub-G0 pick) presented apoptotic population. Minimal number of analyzed cells per sample was 5.000. The results are shown as percentage of cells in apoptosis.

MTT asay

PBMNCs were sowed in 96-well plates (Sarstedt) ($3x10^5$ cells per well, in triplicates), in complete medium. The cells were stimulated with phytohemagglutinin (PHA) (20 µg/ml). After that, 20 µl of nCNC suspension in complete RPMI medium was added, and finally concentration per well was 50 µg/ml–400 µg/ml. Negative controls were the wells with PBMNCs cultivated in medium alone. The mononuclears had been incubated for 72 h at 37°C, with 5% CO_2 and 90% humidity. The wells with nCNCs at the corresponding concentrations in complete medium served as additional controls, whereas the blank controls were those wells with medium alone. The metabolic activity of PBMNCs was detected by a colorimetric assay [20].

After 48 h the plates were centrifuged (900xg, 8 min), followed by removing the supernatants. Following this, the 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT, final concentration 500 µg/ml) (Sigma-Aldrich) solution was added to wells. After a four-hour period of incubation, 10% sodium dodecyl sulphate (SDS) (Merck) was added to each well. The next day, optical density of the evolved colour was analyzed by ELISA reader (BioTek Instruments). The values were presented as the metabolic activity (%) relative to the analogous negative controls used as 100%.

^{3}[H]-thymidine uptake assay

PBMNCs ($3x10^5$/well, in triplicates, of 96-well plates (Sarstedt)) were stimulated (PHA, 20 µg/ml) and cultured alone or with appropriate concentrations of nCNCs (50 µg/ml–400 µg/ml). After 4th day, ^{3}[H]-thymidine (1µ Ci/well) (Amersham) was added for the last 8h. The cells were harvested at the end of the cultivation period. The radioactivity (count per minutes) was detected by scintillation counter (Beckman). The results were presented as percentages compared to the proliferation of cells without nCNC (control, 100%).

Detection of IL-2

The concentrations of interleukin-2 (IL-2) were determined using the supernatants of PHA-stimulated PBMNCs cultures. Appropriate kit (BioLegend) was used in enzyme-linked immunosorbent assay (ELISA) for the measurements of the concentrations expressed in pg/ml. The standard curves were used for assessing the concentrations of IL-2, according to known concentrations of this cytokine.

Statistical analysis

The results are shown as mean ± standard deviation (SD) values of at least three independent experiments (different healthy donors). The data was analyzed using One-way ANOVA to evaluate the differences between the experimental and corresponding control samples. All statistical analysis was performed in GraphPad Prism software (La Jolla, CA, USA). Values at $p<0.05$ were considered statistically significant.

Results

Dose-dependent effect of nCNCs on the cytotoxicity of PBMNCs

The first aim of this study was to investigate cytotoxicity of different concentrations of nCNCs (50-400 µg/ml). Cytotoxicity was assesed by testing necrosis and apoptosis of PBMNCs, as described in materials and methods.

Results presented in Fig. 1A show that nCNCs do not significantly affect the percentage of necrotic cells compared to control. However, when testing apoptosis, it can be seen (Fig. 1B) that the highest concentration of nCNCs (400 µg/ml) induce apoptosis of PBMNCs ($p<0.05$).

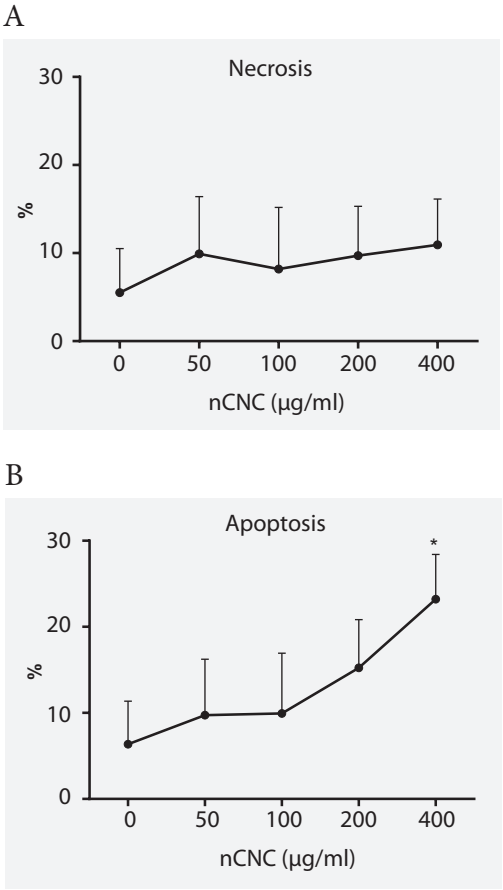

Figure 1. Effect of nCNCs on necrosis and apoptosis of PBMNCs, *p<0.05; **p<0.001 compared to control (n=3)

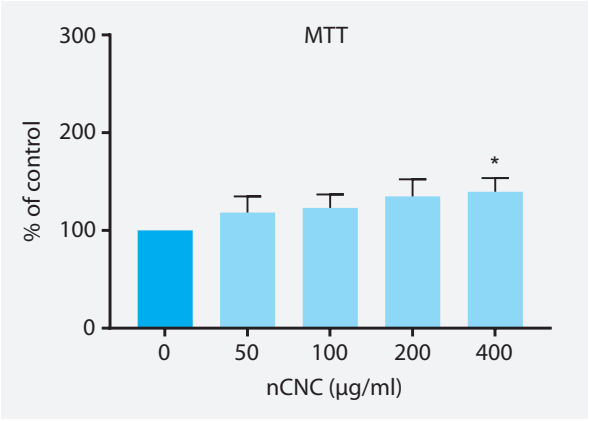

Figure 2. Effect of nCNCs on the metabolic activity of PBMNCs, *p<0.05; **p<0.001 compared to control (n=3)

Effect of nCNCs on the proliferation of PBMNCs

The proliferation of PBMNCs, stimulated with PHA, was measured by MTT and [3H]-thymidine uptake assays. Results presented in Fig.2 show, unexpectedly, that the highest concentration of nCNCs (400 µg/ml) increased the metabolic activity of PBMNCs ($p<0.05$) compared to control. Other concentrations have no significant modulating effect. Opposite to this, there are the results presented in Fig. 3 where significant induction of proliferation was confirmed for lower concentrations of nCNCs (50 µg/ml and 100 µg/ml) ($p<0.05$ and $p<0.001$, respectively).

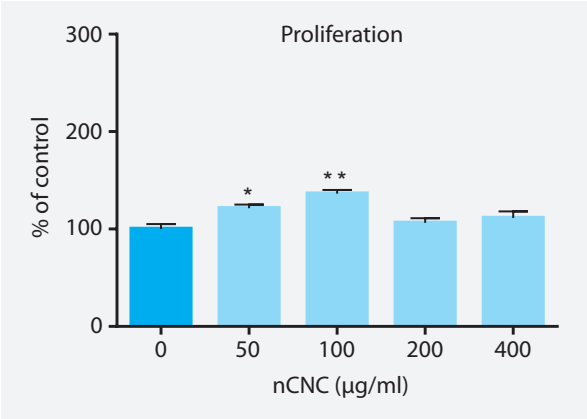

Figure 3. Effect of nCNCs on the proliferation of PB-MNCs in culture, *p<0.05; **p<0.001 compared to control (n=3)

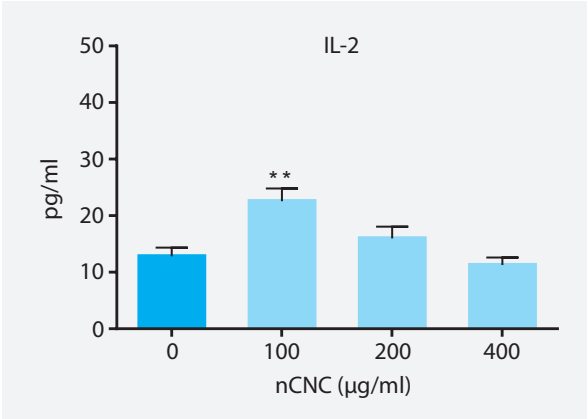

Figure 4. Effect of nCNCs on the IL-2 production in PB-MNCs cultures, *p<0.05; **p<0.001 compared to control (n=3)

The production of IL-2 was determined analysing supernatants of the mononuclear cultures stimulated with PHA. The PBMNCs cultivated with lower concentration of nCNCs (100 µg/ml) produced significant higher level of IL-2 compared to the control cells (p<0.001), whereas other concentrations (200 µg/ml and 400 µg/ml) were non-modulatory (Fig. 4). These results correlate with increased proliferation of PBMNCs.

Discussion

Nanocellulose has become a promising substrate as medical implants [21, 22], cardiovascular applications [23], tissue engineering [24], wound-healing [25], drug excipient and delivery [26], as well as a promising substrate used for antimicrobial purposes [27]. In addition to the typical applications, it has been attempted for nanocell u lose to be used some new biomedical areas such as capture of DNA oligomers [28], cancer targeting [29], biology-device interfaces [30] and biological detection [31].

Compared to other nanocellulose materials, nCNCs possess attractive features including surface chemistry, ability to interact with another polymeric materials, mechanical advantages and comparative affordability and sustainability [32-34]. However, prior to clinical application, it is important to define their biocompatibility and interactions with the context of the constituents of the immune system.

In the study, we investigated the effect of nCNCs on cytotoxicity and their capability to modulate proliferation of T cells using human PHA-activated PBMNCs as in vitro model. Our cytotoxicity research demonstrated that nCNCs were not apoptosis at the highest concentration (400 µg/ml) were concerned. In contrast, a significant stimulating effect on PBMNCs was observed when using lower concentrations. This is the first report on the topic.

Pereira et al. demonstrated that cotton CNCs (above 200 µg/ml) may induce cell death of mammalian fibroblasts in vitro [35]. This phenomenon was confirmed by flow cytometry analysis and increased expressions of cell stress biomarkers (heat shock protein 70.1 and peroxiredoxin1) and apoptosis-associated molecular markers such as BCL-2 associated X protein and B-cell leukemia (BCL). In line with these outcomes, a dose-dependent cytotoxicity of cotton CNCs was observed using an in vitro model of the human epithelial airway barrier [16]. In this 3D triple cell coculture model, epithelial cells had been exposed to the multiwalled carbon nanotubes (MWCNTs), cotton CNCs and crocidolite asbestos fibers (CAFs) for 24h, within the same levels of concentrations ranging between 0.005 and 0.03 mg/ml. It was demonstrated that cotton CNCs induced cytotoxicity but in a lower degree compared to the two other types of nanomaterials. Yanamala and co-workers showed toxicity of CNCs after pulmonary exposure of C57BL/6 mice to two differently processed forms of CNCs [15]. It is of interest that CNCP (powder form) el-

evated biomarkers of tissue damage to a higher extent, whereas greater increases in inflammatory mediators and oxidative stress markers were detected in mice exposed to CNC suspension (10 wt %; gel/suspension).

However, some opposite results were published. For example, no cytotoxic effects of CNCs was confirmed against nine different cell lines (bEnd.3, HBMEC, 7MCF-10A, RAW 264., MDA-MB-468, MDA-MB-231, PC-3, KB and C6) in the concentration range 0-50 μg/mL, aftert 48h [17]. Also, 24-hour exposure to the aerosolised CNCs (0.14 to 1.57 μg/cm^2) did not decrease the viability of cells in the 3D multi-cellular model of the human epithelial airway barrier [18]. In vitro and in vivo cytotoxicity tests with bacterial cellulose (BC), under the conditions and concentrations used, shown no evidence of toxic effect [36, 37]. CNF did not induce necrosis, apoptosis or any DNA damage in different cell lines, unlike chemically modified CNFs [5, 6, 38]. Overall, it seems that many of the observed differences can be ascribed to the wide variety of factors. The significance of appropriate biological systems (cell type), material origin, treatment and characterization, as well as cell exposure doses and time, is particularly accentuated by the seemingly directly opposing results of previous investigations. It seem that, in further studies, clear understanding of these factors is of the utmost importance and is inevitable for testing toxicological behavior of nCNCs and nanocellulose in general. However, the general conclusion is that CNCs, at the similar concentrations used by other authors, are not cytotoxic for human PBMNCs.

In contrast to the cytotoxicity study (apoptosis and necrosis), results obtained by MTT in our experiments, were different. Namely, MTT showed a significant stimulatory effect of cellular proliferation by using the highest concentration of nCNCs (400 μg/ml), as judged by increased optical density in the assay of the PHA-stimulated PBMNCs. This phenomenon could be explained by the interference of nCNCs with the solution of MTT, taking into consideration different findings which were obtained by using the ^{3}[H]-thymidine uptake method.

The most important part of our research referred to the influence of nCNCs on proliferation of T-cells in a PHA-activated model of PBMNCs. In the presence of dendritic cells (DCs) and monocytes, PHA as a T-cell mitogen induces their proliferation. PHA allows transcription of crucial elements for T-cell growth due to interaction with diverse activating molecules on these and antigen-presenting cells (APCs) and T-cell receptor [39, 40]. In this paper, we showed, for the first time, that native CNCs were able to induce T-cell proliferation at lower concentrations (50 and 100 μg/ml). The effect was observed when ^{3}[H]-thymidine uptake assay was applied. It is obvious that the stimulatory effect of nCNCs is an immunological phenomenon depended on a significant increase in IL-2 level. This cytokine, as a crucial T-cell growth factor, is produced by activated T cells. Furthermore, IL-2 has a key role in promoting T-cell activation and proliferation of only those cells that have been stimulated by cognate antigenic interaction [41].

Our findings are not in accordance with the results demonstrated by Moreira et al. who revealed that BC (100 μg/ml-1 mg/ml) inhibited the proliferation of CHO cells and 3T3 fibroblasts after 72h of cultivation [42]. In line with this is slight inhibition of cellular proliferation confirmed on bacterial nanocellulose membranes [43]. Čolić and colleagues confirmed that CNFs (250 μg/ml-1 mg/ml) slightly decreased the proliferation of PHA-stimulated PBMNCs, accompanied by down-regulation of IL-2 [8]. Such properties of CNFs were the result of induction of tolerogenic human DCs, which were able to down-regulate Th1 and Th17 cells, and up-regulate Th2 and Treg [9]. On the other hand, the nanocomposite consisting of CNFs, initially showed inhibitory effect on the proliferation of endothelial and human ligament cells, while exerting a stimulatory effect afterwards [44].

According to the previous results, we suppose that APCs, particularly DCs, predominantly mediate the nCNCs up-regulating effect on activation of T-cells. In this sense, it is worth mentioning that DCs, as first components of the immune system recognizing nanomaterials, have a crucial function in initiating and modulating primary T-cell responses. What is more interesting, these interaction can lead to the initiation of either an immunogenic (inflammatory) or a tolerogenic

(anti-inflammatory) immune responses. It depends on the structural, chemical and biological properties of natural nanoscaled materials, but many other factors may affect this process such as cell types, study models, applied concentrations and exposure time [34, 45-47].

Conclusion

For the first time, we have showed that nCNCs, at non cytotoxic concetrations (50-100 µg/ml), stimulate the proliferative activity of human PBMNCs, which is followed by increased production of IL-2. Further studies are needed for better exploration of the significance of this phenomenon.

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Ethical approval. The Ethics Committee of the Faculty of Medicine in Foca approved the study and informed-consent was obtained from all individual respondents. The research was conducted according to the Declaration of Helsinki.

Conflicts of interest. The authors declare no conflict of interest.

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Celulozni nanokristali indukuju dozno-zavisni efekat na citotoksičnost i proliferaciju humanih mononuklearnih ćelija periferne krvi

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Uvod. Celulozni nanokristali (CNCs) su atraktivni prirodni materijali koji imaju brojne primjene u oblasti biomedicine. Zahvaljujući specifičnim biomehaničkim svojstvima, hemijskim karakteristikama njihove površine, niskoj cijeni i obnovljivosti izvora, pripadaju naprednim nanomaterijalima i predstavljaju odličnu zamjenu za tradicionalne celulozne materijale. Međutim, prije biomedicinske primjene neophodno je provjeriti citokompatibilnost i imunomodulacijska svojstva prirodnih (n)CNCs, koja nisu u potpunosti istražena. Zato je cilj ovog rada bio da se ispita dozno zavisni efekat nCNCs na citotoksičnost i proliferaciju humanih mononuklearnih ćelija periferne krvi (PBMNCs) in vitro.

Metode. PBMNCs, dobijene od zdravih dobrovoljnih davaoca krvi, su kultivisane sa nCNCs. Vijabilnost ćelija je analizirana pomoću protočne citometrije, dok je proliferacija ispitivana na osnovu MTT testa, testa ugradnje [3H]-timidina i detekcijom produkcije interleukina-2 (IL-2).

Rezultati. Rezultati citotoksičnosti pokazuju da nijedna od koncentracija nCNCs (50-400 µg/ml) nije uticala na nekrozu PBMNCs, dok je apoptozu indukovala najveća koncentracija nCNCs u poređenju sa kontrolom ($p<0,05$). Neočekivano, najveća koncentracija nCNCs je povećala metaboličku aktivnost fitohemalutininom (PHA)-stimulisanih ćelija u poređenju sa kontrolom ($p<0,05$). Suprotno ovim rezultatima, manje koncentracije nCNCs (50 µg/ml and 100 µg/ml) su stimulisale proliferaciju PBMNCs ($p<0,05$ i $p<0,001$). To je bilo praćeno povećanjem produkcije IL-2 (100 µg/ml) ($p<0,001$).

Zaključak. Rezultati ukazuju da necitotoksične koncentracije nCNCs moduliraju proliferaciju PBMNCs, fenomen koji do sada nije objavljen u literaturi i zato može biti koristan za dalja istraživanja.

Ključne riječi: celulozni nanokristali, mononuklearne ćelije periferne krvi, kultura, citotoksičnost, proliferacija