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Testing the Hepatotoxicity of Single Walled and Multi Walled Carbon Nanotubes

Carbon nanotubes present a promising new system for efficient drug delivery. The combination of properties holds most significance to the nanotubes' efficiency of molecular transfer by perforation through organic tissue. The use of this method has been associated with the increase of the portion of the drug that has been successfully administered. However, the knowledge on cell toxicity caused by these nanoparticles is remarkably limited. Due to their potential, there is copious research concerning the extent to which the nanotubes can be safely implemented in medicine. In this study the objective was to evaluate the hepatotoxic effects of single walled carbon nanotubes (SWCNT) and multi walled carbon nanotubes (MWCNT). Human liver carcinoma cells (HepG2) were exposed to different concentrations of suspensions of pre-made SWCNT and MWCNT for a duration of 32 h. They were examined under an optical microscope at various intervals after treatment. Viability was tested using an MTT Colorimetric Assay, read at 540 and 670 nm. The results show noticeable cell death only for the experimental group treated with 1 mg/mL SWCNT. While there is apparent agglomeration and anamorphosis in all treated groups, it does not necessarily indicate harmfulness of the nanotubes. From this it might be concluded that in these conditions, regarding the aspect of toxicity, the safety of their use in biological application remains uncertain. It is left to future studies to test possible variations of these results due to different concentrations in the same circumstances.

Introduction

Carbon nanotubes (CNT) are hollow tubular molecules made purely from graphene layers rolled into a cylindrical shape. They can be single walled (SWCNT), composed of one layer of graphene sheet, or multi walled (MWCNT), made up of two or more layers of graphene stacked on top of each other before the tube is formed. In addition, they have proven to be useful in extremely small scale electronic and mechanical applications due to their unique electrical properties, great heat conductivity and notable strength (Endo *et al*. 2008).

Aside from that, carbon nanotubes have numerous potential biological applications, a very important one being drug delivery. In their pure form they are non polar and hydrophobic, thus incompatible with many drug molecules and usually the recipient cells themselves. However, a crucial attribute of these nanoparticles is the ability of their structure to be modified (most often by sidewall or end-of-tube derivatization), in turn increasing their polar solubility or dispersion (Monteiro-Riviere *et al*. 2005)

Although studies show that carbon nanotubes would be an exceptionally efficient method for drug delivery (Bianco *et al*. 2005), the toxicology has not been thoroughly evaluated under environmental and occupational exposure scenarios. A major issue is the health impact to humans exposed to nanomaterials through oral, dermal, or inhalational routes. While there is abundant literature on this topic, it is still insufficient due to the great variety of the structure and modifications of the nanotubes, and the method and route of drug delivery (Monteiro-Riviere *et*

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al. 2005). The antibiotic Amphotericin B, covalently attached with fluorescein isothiocyanate to CNT, has shown to be easily internalised into mammalian cells without toxic effects in comparison with the antibiotic incubated alone (Bianco *et al*. 2005). Similarly, it was observed that cytotoxicity was diminished as SWCNT sidewall functionalization increased, for example, using phenyl-SO_3H and phenyl-SO_3Na additives, and that even at high concentrations there was insignificant damage to cells (Sayes *et al*. 2006), while it was found that most of the short CNTs injected into subcutaneous tissue in rats were in the cytosol of macrophages after 4 weeks, whereas the longer CNTs were still free floating and causing inflammation (Sato *et al*. 2005)

Nonetheless, multitudinous studies suggest that the toxic effects can be too extensive to ignore. SWCNT suspensions (Belanskaya *et al*. 2009) have caused severe neurotoxicity in cultures from both the central and peripheral nervous system in chicken embryos. There have been reports (Jacobsen *et al*. 2008) of genotoxicity of SWCNT in FE1-Mutatrade markMouse lung epithelial cell line, with the majority of cells exposed to CNT exhibiting slower cell proliferation and halting at the G1 phase of the cell cycle. Other toxic effects include but are not limited to oxidative stress (Yang *et al*. 2008), DNA damage (Pacurari *et al*. 2008), and many other types of disturbances (Du *et al*. 2013) that ultimately lead to malfunction or cell death.

With that context, the object of this experiment was to investigate possible toxic effects that might occur upon in vitro treatment of cultures of HepG2 with different concentrations of functionalized SWCNT and MWCNT applied through a 0.9% physiological solution of NaCl, using light microscopy and MTT colorimetric assay to observe inspect morphology and cell viability of the exposed cells.

Materials and Methods

Carbon nanotubes functionalisation. Pre-made pristine single walled and multi walled carbon nanotubes were obtained for the purposes of this research. After unsuccessful attempts of functionalisation using first DMF, aldehyde, and glycine (Georgakilas *et al*. 2002), followed by Triton X-100 surfactant (Rastogi *et al*. 2008), a suspension of nanotubes in PBS was obtained usng sulfuric and nitric acid for the functionalisation process (Osorio *et al*. 2008) The nanotubes were functionalised using 15 mL concentrated H_2SO_4 and 5 mL concentrated HNO_3, with sonication with gradual temperature increase.

Cell culture and treatment. The cells were plated for MTT/Morphology Observation in a 96 well plate, with 90 microliters of cell suspension in each well. The HepG2 cells were plated in 40 wells in total (10^5 cells in each well). Suspensions of both single walled and multi walled carbon nanotubes in PBS were prepared with concentrations of 1 mg/mL, 2.5 mg/mL, and 5 mg/mL. Two control groups were established: one with only cells in medium and another with PBS in addition. Six experimental groups were treated, each with one of the three concentrations of either single or multi walled carbon nanotubes. Every group, control and experimental, had five repetitions.

Cell morphology visualization. The cells' morphology was observed under light microscopy after 8 h, 14 h, 20 h, and 32 h.

MTT assay. The cells were tested with an MTT assay after 32 h of treatment. The medium was poured out and 100 μL of MTT solution (20 mg MTT dye in 40 mL of specific cell medium) were added to each well. The cells were incubated for 3 h at a temperature of 37°C. The MTT solution was poured out and100 μL of a 168 μL 35% HCl in 40 mL isopropanol solution were added to the wells. After incubating for 10 minutes at room temperature, the absorbance was read at 540 nm and 670 nm.

Results

The results show that in comparison with the control group, only 66.8% of cells survived 1 mg/mL SWCNT treatment (Figure 1). No cytotoxicity was observed in treatments with 2.5 mg/mL and 5 mg/mL SWCNT, as well as MWCNT of all three concentrations. While the abundance of cells in the morphology images (Figure 2) reflects the viability report, no significant differences in the shape or behavior of differently treated cells was marked.

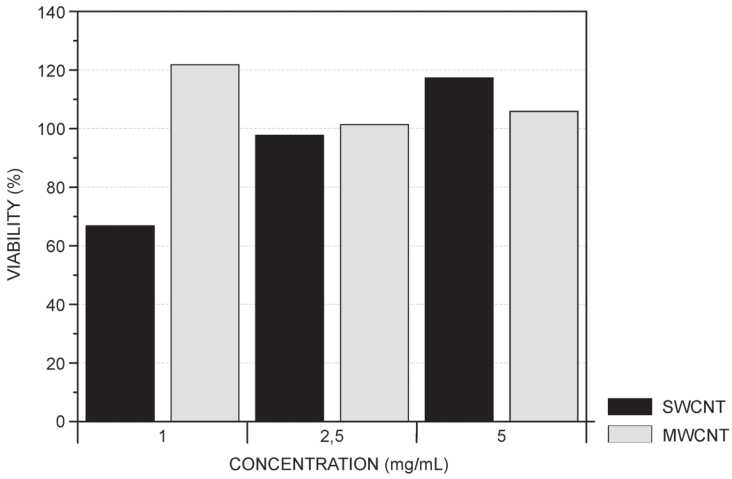

Figure 1. Percent viability of treated cells in comparison with the control group

Slika 1. Procenat vijabilnosti tretiranih ćelija u poređenju sa kontrolnom grupom

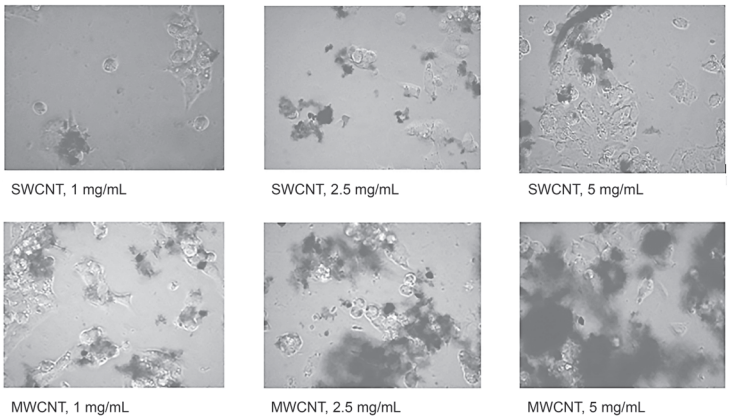

Figure 2. Morphology of the culture, observed under an optic microscope, 20 h after treatment

Slika 2. Snimci tkiva pod optičkim mikroslopom, 20 h nakon tretmana

Conclusion

SWCNT exhibit toxic effects at low concentrations. Due to this, the safety of their use in biological application remains uncertain. While MWCNT appear to have no effect on the cell cultures, thus could potentially be classified suitable for biological use, the lack of toxicity might have a different implication. It is possible that the nanotubes' tendency to aggregate, evident in the obtained microscopy images, inhibits them from penetrating the cells in the first place and having any effect at all. This would implicate that in this set of circumstances, the MWCNT would be an unsuccessful method for drug delivery. SWCNT and MWCNT are currently not applicable for biological application. Further research and optimization is needed for the purposes of this use.

Acknowledgments. Great gratitude to my mentors Nemanja Stanojević and Ivan Milenković, as well as all other assistants from this seminar that supported and assisted this research. Likewise, great gratitude to Spec. Pharm. Biljana Nestorovska-Gjoshevska for setting the idea of this research in motion.

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Ispitivanje hepatotoksičnosti jednoslojnih i višeslojnih ugljeničnih nanocevi

Ugljenične nanocevi su perspektivan sistem za efikasnu isporuku lekova. Osobine koje poseduju, uključujući promenu nano-skale i osetljivost na vezivanje molekula, veoma su značajne za efikasnost prenosa molekulâ kroz organsko tkivo. Primena ovog metoda poboljšava efikasnost isporuke leka, odnosno povećava udeo leka koji je uspešno prihvaćen. Međutim, znanje o toksičnosti ovih nanocevi i dalje je izuzetno ograničeno i trenutno postoje obimna istraživanja o tome koliko se nanocevi mogu bezbedno primenjivati u medicini.

Cilj ovog rada bio bio je da se proceni hepatotoksični efekat jednoslojnih (SWCNT) i višeslojnih ugljeničnih nanocevi (MWCNT). Ćelije jetre (HepG2) bile su izložene različitim koncentracijama (1, 2.5 i 5 mg/L) suspenzija oba tipa karbonskih nanocevi u trajanju od 32 h. Morfologija ćelija ispitivana je pod optičkim mikroskopom 8, 14, 20 i 32 h nakon tretmana.

Vijabilnost je testirana pomoću MTT kolorimetrijskog testa na 540 i 670 nanometara.

Rezultati pokazuju da je samo kod jednoslojnih nanocevi pri koncentraciji od 1 mg/mL zapažena indukovana ćelijska smrt u tretiranim kulturama. Iako postoji očigledna aglomeracija i anamorfoza u svim tretiranim grupama, ona nužno ne ukazuje na štetnost. Iz ovoga se može zaključiti da u ovim uslovima, s aspektra toksičnosti, sigurnost upotrebe nanocevi u biološke svrhe nije dovoljno očigledna. U nastavku istraživanja trebalo bi ispitati uticaj drugih koncentracija u istim uslovima.