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Research Article

TOXICITY ASSESSMENT OF CARBON NANOTUBES ON ERYTHROCYTE MORPHOLOGY AND LYMPHOCYTES *IN VITRO*

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ABSTRACT

Objectives: Carbon nanomaterials have been used in many biomedical applications due to its unique physical properties. Functionalization of carbon nanotubes (CNT) could improve the physical properties, but pharmacokinetics and toxic effects of pristine and functionalized CNTs, are not well defined. In this study, the toxicity of the pristine and hydroxyl group functionalized Multi-Walled CNT (MWCNT) compared.

Methods: MWCNTs was interacted with human blood. The induced morphological changes in the erythrocytes and cytotoxicity were observed at different concentrations. A thin blood smear was prepared, and the erythrocyte images were obtained using a digital microscope. MTT assay used to assess the cytotoxicity.

Results: The result indicate that the pristine MWCNT caused more toxic effects than –OH functionalized MWCNT, which was assessed in terms of changes in the morphology of the erythrocytes and cytotoxicity caused to the lymphocyte cells.

Conclusion: The functionalization of MWCNT could reduce the hemotoxicity and improves the biocompatibility.

Keywords: Carbon nanotubes, Cytotoxicity, Echinocytes, Erythrocyte, Multi-walled carbon nanotubes, Lymphocyte.

INTRODUCTION

Nanotechnology is a multidisciplinary field widely applied in science and engineering techniques that could focus on the measurement and control at a molecular level. Remarkable methods of nanoparticle applications in the area of biomedicine and biotechnology have been developed enormously [1-3]. A carbon nanotube (CNT) possesses unique properties that are creating an interest in the field of biomedical research [4]. Many types of research proposed different applications of CNT in various area of biomedical research [5]. Due to excellent electronic, optical properties and acoustic response of CNTs facilitating its use in molecular imaging such as molecular magnetic resonance imaging, magnetic resonance spectroscopy, optical bioluminescence, optical fluorescence, targeted ultrasound, single photon emission computed tomography, and positron emission tomography [4,6]. Single-walled and multi-walled (SWCNT and MWCNT) forms are considered for applications in biomedical engineering [7]. The toxicological response depends on the form, manufacturing process, route of exposure and dosage [8]. So far, many *in-vivo* and *in-vitro* studies have reported the potential toxicity of CNTs by different methods. CNTs induced pulmonary toxicity [9], genotoxicity [8], hemotoxicity, hepatotoxicity [10], induced oxidative stress, DNA damage, carcinogenicity, and induced inflammatory factors [11]. Carbon nanomaterials are also administrated intestinally for medical imaging, drug delivery, biosensing, and coating material of implants [12]. Furthermore, there is a possibility of unintended exposure of carbon nanomaterials to the environment [13]. Whether intended or unintended exposure of carbon nanomaterials, toxic effects caused to human health is an important concern.

METHODS

Chemicals

Multi-walled CNT Type 3 (Pristine) (OD: 10~20 nm Length: 10-30 μm Batch number: T8380147) was procured from Sisco Research Laboratories Private Limited, Mumbai. It was dispersed with 1.5% Tween 20 surfactant obtained from Sigma-Aldrich, USA. A colloidal mixture of MWCNT with tween 20 (1.5%) was prepared by ultrasonic vibration using 20 KHz ultrasonic processors, with a maximum output of 750 W for 30 minutes (Sonics, USA). The sonication was at 10 seconds pulses off and 60 seconds pulses on and at 40% amplitude in each cycle. Ice cubes in a beaker were used to keep container to avoid any effect due to heat generation. Multi-Walled CNT Type 15 (–OH functionalized) with an outer diameter ranging from 30~50 nm, Batch number: T 8371597 was procured from Sisco Research Laboratories Private limited, Mumbai. CNT dispersion prepared 30 minutes before the experiments using ultrasonicator.

Samples preparation

Human blood collected from healthy individuals, between 25 and 30 years was chosen for the study. The exclusion criteria for the donor were strictly followed as non-smokers and non-medication therapy. For erythrocytes morphology, thin blood smear was prepared from the heparinized blood. Previously, the blood cells were incubated with pristine and –OH functionalized MWCNT at 37°C for 24 hrs. The blood smear was made using wedge slide techniques [14]. The erythrocyte images were obtained using Leica DM 2500 digital microscope. The scoring of abnormal erythrocytes (echinocyte) was counted out of 1000 cells.

Cytotoxicity (MTT assay)

After 24 hrs of incubation, the human lymphocyte cells were counted and 1 × 10^4cells were seeded in 96-well culture plates with different concentration of pristine and –OH functionalized MWCNT (5, 25, 50 and 100 μg/ml) and, incubated at 37°C for 24 hrs. After incubation, cells were treated with 5 mg/ml solution of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) at 37°C for 4 hrs in 5% CO_2 incubator. Then, 50 μl of dimethyl sulfoxide was added to solubilize the formed formazan. The number of viable was read by Microplate Absorbance Reader (BioTek) at 532 nm. The human lymphocyte cells untreated with CNT were taken as a control [15].

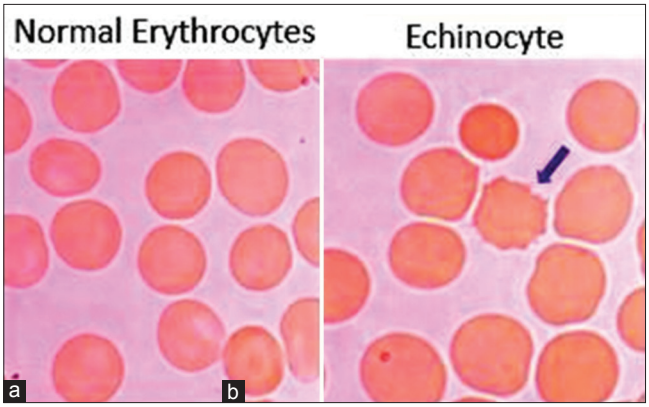

Fig. 1: (a and b) Normal and abnormal erythrocytes (echinocyte)

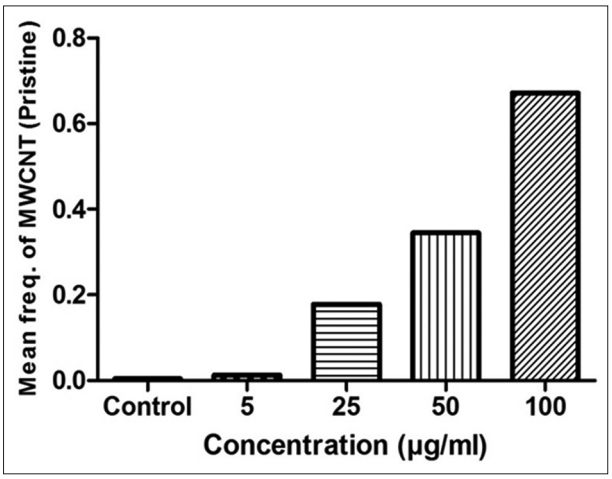

Fig. 2: Mean frequency of multi-walled carbon nanotubes (pristine) in erythrocytes morphology

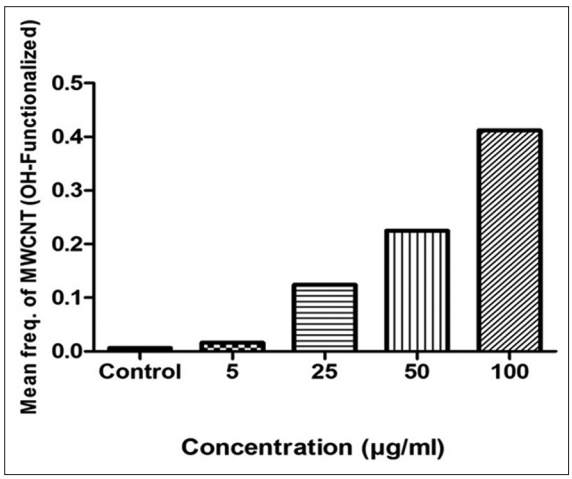

Fig. 3: Mean frequency of multi-walled carbon nanotubes (–OH functionalized) in erythrocyte morphology

RESULTS AND DISCUSSION

Erythrocytes morphology

The blood sample-interacted with MWCNT influences the erythrocyte morphology and induced echinocytes (Fig. 1). The blood sample-interacted with MWCNT (pristine) have more number of crenated cells (echinocyte) than the cells treated with a hydroxyl group functionalized CNT. (Figs. 2 and 3). It may be due to the aggregation of MWCNT with the plasma membrane of erythrocytes.

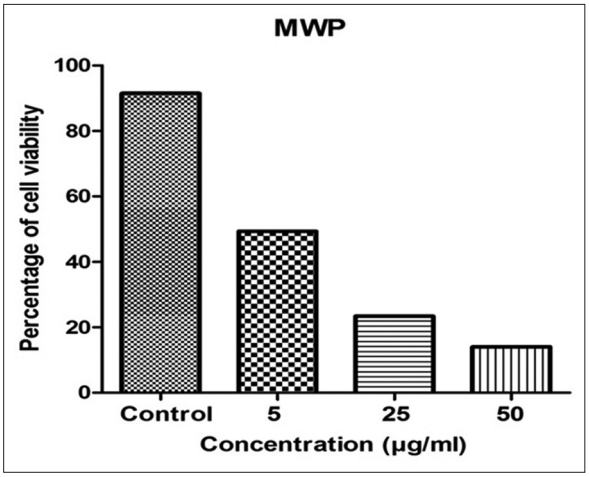

Fig. 4: The mean frequency of cell viability in multi-walled carbon nanotubes (pristine) treatment using MTT assay

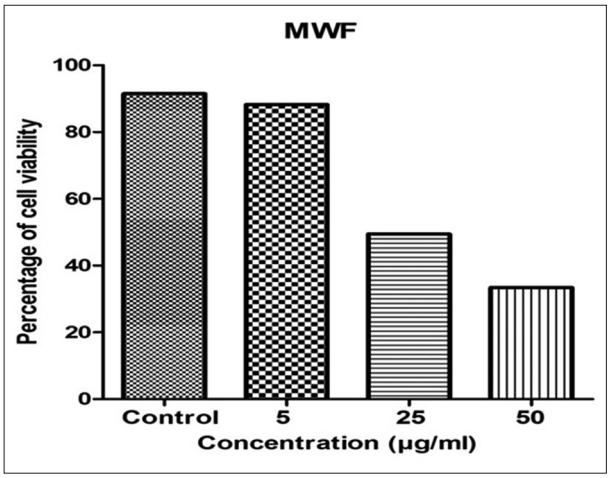

Fig. 5: The mean frequency of cell viability of multi-walled carbon nanotubes functionalized using MTT assay

Erythrocytes are major cellular components of the blood. The flow resistance of the blood in microvessels is directly related to shape transformation of erythrocyte in blood circulation [16]. The nanoparticle can attach around the erythrocyte. The nanoparticles modify the native properties of erythrocyte membrane and lead to morphological changes and cell death [17,18]. The morphology is important as any for deformability of erythrocytes may affect the blood rheology in cardiovascular system [19]. Hence, changes in morphology that might affect the blood flow through the cardiovascular system were analyzed.

Cytotoxicity (MTT assay)

The cytotoxicity was observed in human lymphocytes after 24 hrs of interaction with CNT using MTT. MTT assay has been frequently used for determining the *in vitro* cytotoxicity of CNT in cell culture experiments [20]. Results of MTT assay showed a significant reduction in cell viability compared to control in a dose-dependent manner. At 5, 50, and 100 µg/ml of the Pristine and –OH functionalized MWCNT showed a significant reduction in percentage cell viability ($p < 0.001$). Interestingly, the pristine and –OH functionalized MWCNT have a similar significant decrease in cell viability and the lowest reduction in cellular viability was observed with –OH functionalized MWCNT. The pristine MWCNT were found to be more toxic to human lymphocytes (Figs. 4 and 5).

The MWCNT (pristine) and –OH functionalized MWCNT both damages the cell membrane. Interaction with human bronchial cells shows different effects on cell viability. The authors have reported that the functionalized MWCNT – COOH shows more cytotoxicity in the tested bronchial cells. The functionalized MWCNT causes genotoxicity without inducing an inflammatory response, but pristine induces the production of inflammatory factors [21]. Our study reveals that the functionalized CNT were found to cause lower toxic effects than the pristine form of CNT. Since the lymphocytes have specific immune response to infectious microorganisms and foreign substances it is considered as one the important immune cells [22]. Pristine and functionalized MWCNT both are toxic although pristine shows more cytotoxicity in human lymphocyte cells.

CONCLUSION

The MWCNT (pristine) induced more changes in the erythrocyte morphology and shows more cytotoxicity in human lymphocyte cells than the –OH functionalized MWCNT. Our study suggests the functionalization of CNT could reduce the toxic effect but further studies on immune response, concentration, and membrane penetration is essential before using them in medical applications.

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