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CYTOTOXIC STUDY OF CARBON NANO TUBES

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ABSTRACT

Use of nano carbon in living system has always been a topic of debate. There are two schools of thought (i) believes that nano carbon is inert and can be used in living system whereas the other (ii) feels that they are toxic to living system. Hence, cytotoxicity test using T lymphocytes collected from peripheral blood samples of human cells was performed. The data indicated that the concentration of Multi walled Carbon Nano tube (MWCNT) is an important criterion in causing inhibition to proliferation. It is found that higher concentration of Carbon Nano Tube (CNT) caused decreased cell growth in cultured human blood cells. Though it is still too early to establish CNT for clinical use.

KEYWORDS

Carbon Nano Tubes, Lymphocytes cells, Cytotoxicity and Proliferation.

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INTRODUCTION

Since several years' large number of studies has been done to understand the toxic effects of carbon nanotubes. The conclusions of the reports depend on the type of nanotube materials. Cell culture experiments and *in vivo* studies shows carbon nanotubes are not toxic if properly functionalized (Kam *et al*, 2004[1], Dumortier *et al*, 2006[2], Schipper *et al*, 2008[3] and Wu *et al*, 2008[4]). On the other hand, raw carbon nanotubes were shown to be toxic to mice after inhalation into the lung (Lam *et al*, 2004[5], Warheit *et al*, 2004[6], Shvedova *et al*, 2005[7] and Muller *et al*, 2005[8]). Some of the research work showed that MWNTs if not functionalized, may

cause carcinogenic risk in mice (Poland *et al*, 2008)[9]. It is thus critical and urgent to clarify the toxicity issue of carbon nanotubes. Functionalized CNTs have shown to be nontoxic.

***In vitro* toxicity of carbon nanotubes**

The toxic study of carbon nanotubes in cell culture is still controversial. While inhibition of HEK 293 cell proliferation after exposure to SWNTs was reported by Cui *et al*, (2005)[10]. Ding *et al*, (2005)[11] observed that MWNTs induce cell cycle arrest and increase apoptosis/necrosis of human skin fibroblasts. Sayes *et al*, (2006)[12] further reported that the toxicity of CNTs was dependent on the density of functionalization, with minimal toxicity for those heavily functionalized with the highest density of phenyl-SO3X groups. CNTs without proper functionalization have a highly hydrophobic surface, and hence may aggregate in the cell culture and interact with cells by binding to various biological species, including proteins, via hydrophobic interactions, and induce certain cell responses such as cell toxicity. Surfactants, present in excess in the CNT suspensions, are known to be highly toxic to cells (Dong *et al*, 2008)[13]. The metal catalyst content in CNTs should also be considered when the toxicity of carbon nanotubes is investigated (Plata *et al*, 2008)[14]. Moreover, proper assays must be employed in toxicity tests to avoid interference of carbon nanotubes with the assay reagents (Casey *et al*, 2007[15], Worle-Knirsch *et al*, 2006[16]). Cells exposed to SWNTs, PEGylated by various PL PEG amphiphiles, exhibited neither enhanced apoptosis/neurosis, nor reduced proliferation of various cell lines *in vitro* [Liu *et al*, 2007[17] and Kam *et al*, 2005[18]). Carbon nanotubes covalently functionalized by 1, 3-dipolar cycloaddition developed by Prato *et al*, also appeared to be safe to the tested cell lines, including primary immune cells (Wu *et al*, 2005[19] and Dumortier *et al*, 2006[2]). Carbon nanotubes with a biomimetic coating engineered by Bertozzi et al were also nontoxic to cells (Chen *et al*, 2004[20] and Chen *et al*, 2006[21]). Several other independent groups also reported that CNTs coated by DNA, amphiphilic helical peptides and serum proteins were not toxic to cells (Heller *et al*, 2005[22], Chin *et al*, 2007[23] and Yehia 2007[24]). In very recent work, Jin et al. discovered that SWNTs taken up by cells via endocytosis exited cells through exocytosis without affecting the viability of cells (Jin *et al*, 2008)[25].

MATERIAL AND MEHODS

MWCNT - These MWCNTs were synthesized by CVD method using Fe/Ni as catalyst having 95% purity. It has a diameter of 30-40nm and length of 5-50μm. It has <2% amorphous carbon and < 0.2% ash. The surface area of MWCNT was 300m^2/g, whereas its thermal conductivity was 1812+300W/m.K. It was procured from Monad Nanotech Pvt Ltd. It was purified by nitric acid.

Source of lymphocytes

Blood samples were taken from 7 healthy patients who were not on medication nor had a history of any illness in the past 2 years.

Medium Used for Culturing Lymphocytes

Was Ficoll Hapaque, RPMI- 1640 Medium, Foetal Bovine Serum (FBS) (from Sigma-Aldrich) and Gentamycine (from Nicholas Piramal).

Reagent tried for Lymphocyte Proliferation assay

Was Alamar Blue (AB) (from Biosource).

Lymphocyte Proliferation assay

Was done to test the Cytotoxicity of CNT blood were collected in Heparin tubes. Peripheral blood (PB) T lymphocyte was isolated by Ficoll h-paque separation method. Cells were then culture in RPMI- 1640 media supplemented with FBS and gentamycine making the concentration of cells 1 million/ml. Incubation of cells was done by adding 20μl of nanomaterials to 180μl of cell suspension at 37^0C in 5% CO_2 atmosphere for 72 hours. The final concentration of added nanomaterials was 10, 25, 75, 100mg/ml. After 72 hours of treatment with MWCNT, 20μl of Alamar Blue was added in each well and incubated for 24 hours. Reduction in Alamar Blue showed the percentage proliferation of lymphocyte. The proliferation of lymphocyte was calculated by formula

Percentage reduction in Alamar blue,

$$= \frac{(E2 \times Ab1) - (E1 \times Ab2) \times 100}{(R1 \times N2) - (R2 \times N1)}$$

Where:

E1 = molar extinction coefficient (E) of oxidized Alamar Blue (Blue) at 540nm

E2= E of oxidized Alamar Blue at 630nm

R1 = E of reduced Alamar Blue at 540nm

R2= E of reduced Alamar Blue at 630nm

Ab1 = absorbance of test wells at 540nm

Ab2 = absorbance of test wells at 630nm

N1 = absorbance of negative control well (media plus Alamar Blue but no cells) at 540nm

N2 = absorbance of negative control well (media plus Alamar Blue but no cells) at 630nm

Effect of MWCNT on Lymphocyte proteins by SDS PAGE

Pellet of the suspension of cells by centrifugation at 2500 x g for 10 minutes. Supernatant was discarded. 1ml of Mammalian Protein Extraction Reagent (M-PER) (Thermo scientific) was added to each pellet. After shaking for 10 minutes debris were removed by centrifugation at 14000 x g for 15 minutes. The supernatant were then analyzed on 8% SDS PAGE.

Data analysis

Total IgE serum level was analyzed by Mann-Whitney Test. Cell growth data were analyzed by applying the paired sample t-test. In all cases, P value lower than 0.01 were considered as statistically significant. The statistical tests were performed by SPSS 15 software.

RESULTS

The total IgE serum level was 184.57$\pm$31.61

The viability of freshly isolated lymphocyte suspension assessed by trypan blue staining was 96$\pm$6%.

Concentration of MWCNT used were1, 2.5, 7.5, 10µg/ml. It shows that there is decrease in T lymphocyte cells with increasing concentration of MWCNT as compared to control.

Significant difference for treated cells with MWCNT vs. untreated cells (control) group; *P<0.05, **P<0.01; pair sample t-test.

Lymphocytic protein is more to be affected by the exposure to pathological protein hence it was decided to assess lymphocytic protein by SDS-PAGE analysis of lymphocyte treated with different doses of MWCNT.

Discussion

Time dependent and dose dependent toxicity of SWCNT has been shown by (Cui et al 2005) on proliferation of kidney epithelial cell whereas similar findings were obtained by MWCNT on skin epithelial cells (Monteiro-Riviere *et al*, 2005)[26]. Zeni *et al*, (2008)[27] have shown that SWCNT concentration of upto 10µg/ml filled to induced DNA damage but higher concentration like 25 and 50µg/ml caused decreased cell growth in cultured human blood cells.

In the interesting finding by Ryan *et al*, (2007)[28] have shown that human mast cells and peripheral basophil which are involved in allergic response where inhibited by fullerene and Carbon Nano materials.

Since our efforts have been studying the in vitro toxicity of MWCNT, we feel that to address the possible side effects of CNTs on human health and our environment, researchers have investigated the toxicology of CNTs in animal models. Non functionalized raw CNTs have been intra-tracheally (IT) instilled into animals, showing obvious pulmonary toxicity including unusual inflammation and fibrotic reactions due to the aggregation of hydrophobic raw CNTs in the lung airways (Lam *et al*, 2004[5], Warheit *et al*, 2004[6], Shvedova *et al*, 2005[7] and Muller *et al*, 2005[8]). Those results suggest that aerosol exposure of raw CNTs in the workplace should be avoided to protect human health. Nevertheless, toxicities observed by intratracheal instillation of large amounts of raw CNTs may have little relevance to the toxicology profile of functionalized soluble CNTs for biomedical applications, especially when they are administered through other routes such as intraperitoneal (IP) and intravenous (IV) injections, by which lung airways are not exposed to CNTs.

Furthermore, length dependent pathogenicity was observed, as no obvious toxic effect was observed for shorter and smaller MWNTs (length 1- 20µm, diameter 10 - 14nm), indicating that the toxicology profiles of CNTs may significantly differ between CNTs of various sizes (diameter and length). It is worth noting that functionalized SWNTs used in typical biomedical research have length 50-300nm

and diameter 1-2nm, which are entirely different from the geometry of MWNTs used by Poland *et al*[9].

To fully address the toxicity, concern of CNTs, further investigations including animal models other than mice and on larger scales, are still required. Moreover, the interactions between administered CNTs and the immune complement system, whose activation is an important first line of defense against foreign species, especially microbes, require more attention (Salvador-Morales *et al*, 2006)[29]. Moreover, increased efforts are needed not only from the chemical aspect i.e., further optimizing CNT surface chemistry and geometry for improved biocompatibility, but also from those with biological expertise, to systematically study the complete CNT toxicology profile in different animal models with different routes of administration.

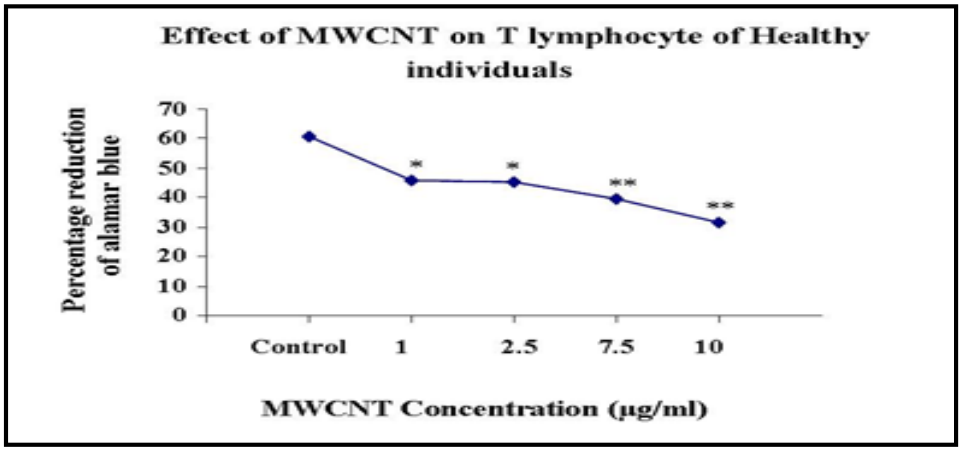

Figure No.1: Graph showing effect of MWCNT on T lymphocytes of healthy persons

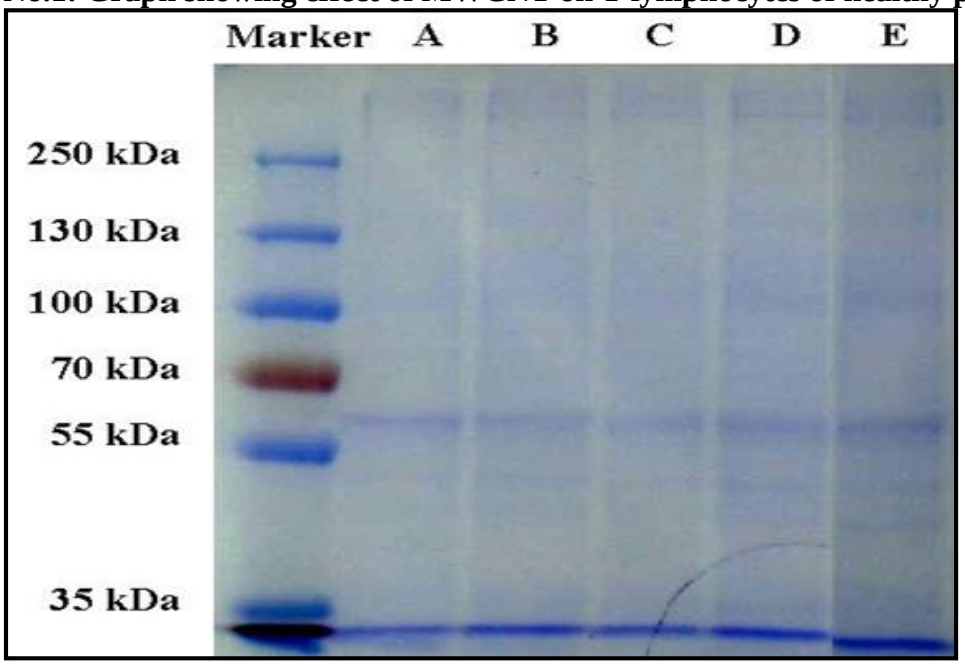

Figure No.2: Effect of different concentration of MWCNT on the protein of the T lymphocyte (A) 10µg/ml MWCNT (B) 7.5µg/ml MWCNT (C) 2.5µg/ml MWCNT (D) 1µg/ml MWCNT and (E) Control (no MWCNT)

CONCLUSION

With the advent of nanomaterials; interdisciplinary research has taken a big leap. The present work was a small effort to enter in this magnificent field of science having immense possibilities. Application of Nanotechnology in medicine found its way in late 1970s (Birrenbch and Speiser 1976[30] and Kopf *et al*, 1976, 1977)[31,32]. Initially polymers having capacity to form micelle, and later polymers having dendritic nature were tried for this purpose.

After going through the books and published papers it was realised that apart from nano-sized organic polymers; nano-metals and carbon nano materials are also being considered as possible drug delivery vehicle e.g. Fullerenes and CNT (Bianco *et al*, 2005)[33].

In the present work the effort was focussed on trying the use of nano materials for drug delivery and carbon nano material was chosen, because CNMs have diverse tunable physical properties as a function of their size and shape due to strong quantum confinement effect and large surface to volume ratio. CNTs are hollow, tubular, caged molecules Because of there properties they have been proposed as light weight material as a nano scale containers for molecular drug delivery. Moreover, Bianco *et al*, (2005)[33] showed that CNTs are adept at entering the cytoplasm as well as nuclei of cells hence can be used as nano-delivery vehicle.

Use of nano carbon in living system has always been a topic of debate. There are two schools of thought (i) believes that nano carbon is inert and can be used in living system whereas the other (ii) feels that they are toxic to living system. Hence, cytotoxicity test using T lymphocytes collected from peripheral blood samples of human cells was performed. The data indicated that the concentration of MWCNT is an important criterion in causing inhibition to proliferation. Dose dependent toxicity of SWCNT has aslo been shown by Cui *et al*, (2005)[10], Monteiro-Riviere *et al*, (2005)[26] and Zeni *et al*, (2008)[27]. They all found that higher concentration of CNT caused decreased cell growth in cultured human blood cells.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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