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Organ Specific Toxicity of Single and Multi-Walled Carbon Nanotubes: A Review

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Abstract *Carbon nanotubes are one of the most widely investigated carbon structures because of variety of physicochemical features offered by them. Their dimensions, surface chemistry and functionalization opportunities make them exceptional carrier for targeted drug delivery gene therapy, diagnosis and cell imaging. Both single and multi-walled CNTs have been functionalized for therapeutic applications. However, these structures have been associated with potential in vitro and in vivo cytotoxic effects. Such toxicities have been described to involve both cellular and subcellular mechanisms. Major organs that have shown significant CNTs related toxic effects include lungs, brain, heart, kidney, liver and skin. This review focuses on the potential toxicological effects of single and multi-walled carbon nanotubes and functionalization on these organs and associated mechanisms of toxicity.*

Key Words: Carbon Nanotubes, Toxicity, Organ Specific, Mechanisms, Functionalization

Introduction

Discovery of sp2 hybridized carbon structures opened a fascinating opportunity for intensive investigation on carbon based structures [1]. Interest in research based on carbon nanotubes (CNTs) developed with their initial accidental observation under transmission electron microscope while studying surface chemistry of graphite electrode [2]. These small carbon structures with their breakthrough discovery became one of major research interest among scientists. Uniqueness of carbon nanotubes has been attributed to their helical structure that furnishes them unique electronic features [3]. In case of carbon nanotubes the graphene cylinders can be appear in two different arrangements. Multi walled carbon nanotubes (MWCNTs) are known as mesoscale graphene systems that appear as large structures with collection of two or more concentric graphene tubes (like rings in tree) [4]. In contrast, single walled carbon nanotubes (SWCNTs) have been described as hollow, one dimensional cylindrical quantum wires, where sp2 carbon graphene sheets are rolled into seamless cylindrical form with high diameter to length ratio [5, 6]. Several methods have been established for synthesis of carbon nanotubes on large scale including arc discharge method [7], laser vaporization method [8] and carbon arc method [9]. However their synthesis in a controlled manner still remains a challenge [10].

The unique structural feature of CNTs offer several ways for their functionalization to achieve specific objectives. Caps at the terminal ends of carbon nanotubes have proven to be more reactive as compared to middle cylindrical section and thus propose a great site for functionalization [10].

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Functionalization of carbon nanotubes has also been associated with reduction in related cellular toxicity [11]. All these features make CNTs an ideal candidate for use in targeted drug delivery and diagnosis [12]. Surface of CNTs can be conjugated with several different functionalities to achieve different functions at same time such as targeting, imaging and drug delivery [13]. Such functionalized CNTs have been explored for wide range of therapeutic applications such as diagnosis and cellular imaging [14], translocation of DNA in cells [15, 16], siRNA mediated gene silencing [17, 18], treatment of infectious disease [19, 20], vaccination and immunization[21, 22] and chemotherapy [23-25]. Besides such a broad therapeutic spectrum of carbon nanotubes a very scarce data is available relative to their possible toxic aptitude. Some studies have compared the relative toxic potential of single walled and multi walled CNTs while some researches demonstrate the effect of functionalization on viable cytotoxicity. This review aims at describing the possible organ related toxicity of SWCNTs and MWCNTs studied both *in vitro* and *in vivo*.

Pulmonary Toxicity of Carbon Nanotubes

Due to unique properties of CNTs i.e. light weight, small size, highly organized structure and electrical, chemical and thermal properties, they are attractive candidates for biomedical application[26]. But there are certain limitations encountered during their use and one of these limitations is the toxicity associated with their delivery through pulmonary route of administration. James B. Mangum and his colleagues conducted a study on effect of SWCNTs on lungs of rats after 21 days exposure following inhalational route of administration[27]. Histopathological evaluation of cells revealed that single-walled carbon nanotubes do not cause lung inflammation, but the interstitial fibrosis of alveolar cells was observed along with increased level of platelet derived growth factor, connective tissue growth factor and transforming growth factor β1. Davoren and coworkers conducted a study to observe in vitro toxicity of SWCNTs on human A549 lung cells i.e. a human lung carcinoma epithelial cell line, they utilized SWCNTs with diameter range of 0.8-1.2nm as treatment and Quartz powder with particle size distribution of 0.35-3.50µm, TEM studies confirmed the morphological alterations in A549 lung cells after SWCNTs exposure and increase in the lamellar bodies at the exposure site [28]. Following injection into peritoneal cavity, carbon nanotubes causes the inflammation and fibrosis in the peritoneum cavity [29]. This gave the idea that carbon nanotubes can accumulate in the sub-plural cavity causing the inflammation and fibrosis of lung cells. Carbon nanotubes may initiate inflammation, granulomas formation and fibrosis which may lead to genetic toxicity. In a study, Robert R. Mercer and coworkers suggested that SWCNTs resided temporarily in alveolar macrophages and then migrated through alveolar epithelium into interstitial space, while, the MWCNTs were highly concentrated in alveolar macrophages having high penetration in alveolar epithelium and visceral pleura resulting in formation of granulomatous lesions in the alveolar airways which accounted for about 8-20% of lung burden [30]. Ma-Hock and his colleagues worked on comparative analysis of inhalation toxicity of multi-wall carbon nanotube, graphene, graphite nanoplatelets and low surface carbon black [31]. They used carbon based structures and administer them to selected groups of mice in submicron range. Regarding the toxicological response, MWCNTs showed the greater response i.e. inflammation at specific site which may depict size distribution that may be involved in that particular response.

In another study, Park and associates performed experiment in mice following intra**-**tracheal administration and on RAW264.7 cells i.e. a murine peritoneal macrophage cell line to check the toxicity of pristine SWCNTs and suggested that P-SWCNTs induced the acute inflammatory response in lungs of mice and autophagy and ER- stress in the macrophages and the physicochemical properties e.g. surface charge and size of CNTs involved in the ultimate autophagy and immune response [32]. Pro-inflammatory mediators especially IL-1 is involved in the inflammation and pulmonary fibrosis by activation of fibrogenic pathway on surface of macrophages which ultimately activates TNF which was observed after the administration of CNTs to murine lung cells [33]. Ursini and coworkers studied the *in vitro* comparative cytotoxic and genotoxic response in A549 lung cells and BAES-2B cell lines to pristine and COOH functionalized MWCNTs. It was concluded that bronchial cells were more susceptible to cytogenotoxicity of COOH functionalized CNTs and to inflammatory response of pristine CNTs while alveolar cells were liable to cytogenotoxicity of pristine CNTs and inflammatory response of COOH functionalized CNTs [34].

The long-term treatment with long and short single walled carbon nanotubes resulted in the fibrosis and epithelium loss. As the long SWCNTS deposits at

the terminal portion of bronchioles while the short SWCNTs reached alveoli and both produced the additive chronic inflammatory response as suggested by a 104-week pulmonary toxicity study involving intra-tracheal administration in rats [35]. While the intra-tracheal administration of SWCNTs of biocompatible nanoscale minimizes *in vivo* pulmonary toxic potential of CNTs[36]. Association exist between the size and surface characterization with the possible toxic lung response i.e. alveolitis and fibrosis after the long-term administration of carbon nanotubes with certain dimensional scale [37].

Surface functionalization of carbon nanotubes is done for utilizing them in biomedical application, usually covalent and non-covalent approaches for surface binding and coating of MWCNTs are utilized [38]. In a study, toxic effects of functionalized multi walled carbon nanotubes were evaluated on murine lung cells. They functionalized the carbon nanotubes with carboxyl, polyethylene glycol, hexane diamine, amine and polyethyleneimine groups and exposed myeloid cell line, THP-1 and BEAS-2B cells i.e. an immobilized human bronchial epithelial cell line. The results of study suggested that all MWCNTs except COOH tubes increased the level of IL-1β, TGF-β1 and PDGF-AA in the treatment cells and COOH nanotubes caused slightly elevated level of these factors which results in cytokine production and PEI-MWCNTs induced significantly a higher response to pro-fibrogenic factors which mediated cytokine production and initiated an inflammatory response. The factors involved in producing that pro-fibrogenic response were surface charge and length of CNTs.

The mechanism reported for cytotoxic response induced by carbon nanotubes was generation of ROS which was responsible for oxidative stress and activated a signaling pathway in phagocytic cells resulting in inflammatory response [39]. The surface hydrophilicity and coating of CNTs with biomolecules has impact on cellular uptake and cytotoxic response of carbon nanotubes[40]. AKT–TSC2–mTOR signaling pathway which involves mTOR i.e. a signaling protein normally exist in phosphorylated form but by certain signaling molecules, it gets activated and causes autophagy of cells; this pathway contributes to the lung's cell injury by functionalized SWCNTS, like -COOH functionalized carbon nanotubes which activates this autophagy signaling pathway results in cell injury[41]. Acute phase pulmonary response can be related to the physicochemical properties of MWCNTs. Pulmonary exposure to MWCNTs initiated a dose dependent acute phase pulmonary toxic response; i.e. at low doses with short length. MWCNTs were also observed to be responsible for mild toxic response of inflammation which reduces with time but the administration of high dose led to high degree of inflammation and lung fibrosis [42].

Neurotoxicity of Carbon Nanotubes

The increased biomedical applications of carbon nanotubes evaluated a great concern regarding the toxic potential of single walled as well as multi-walled carbon nanotubes[43]. Wang and his coworkers investigated toxicity of SWCNTs to PC12 cells and observed that carbon nanotubes increased the oxidative stress in brain cells resulting in production of certain peroxides, superoxide and oxidative enzymes causing detrimental effects to neuronal cells [44]. Scientists have also studied to the effect of cytoprotective and neuroprotective vitamin E on toxic impact of CNTs to the neuronal cells. They suggested that administration of vitamin E during the treatment with single walled carbon nanotubes induces a neuroprotective effect on neuronal cells by reducing the level of oxidative enzymes involved in the generation of reactive oxygen species and elevating oxidative stress level [45]. Single walled carbon nanotubes induce neurotoxicity at biomedical, cellular and gene level. Mechanism involved in neuronal cell damage is the generation of oxidative stress while functionalization with organic molecules like PEGylated carbon nanotubes reduces the toxic effects to neuronal cells and induces low neuronal cell damage than the uncoated single walled carbon nanotubes. This effect was observed when a certain dose of both coated and uncoated SWCNTs were administered to PC12 cells used for the evaluation of *in vitro* neurotoxicity in mice [46]. To access the effect of PEGylated SWCNTs on the rat hippocampus, a study was conducted which illustrated that at low and intermediate dose of SWCNTs, certain impaired retrieval in the contextual fear memory of rat was observed [47].

In case of multi-walled carbon nanotubes, the neuronal cell damage or neurotoxicity is independent to the dose or type of material, it majorly dependent on the inherent potential of MWCNTs to reside in the macrophage and produces a toxicological response[48]. The functionalized multi-walled carbon nanotubes in the brain cells i.e. microglia, astrocytes and neurons resulted in the intermittent release of inflammatory mediators and produces a neurotoxic response following cortical stereotactic administration [49]. The impurities present in the

nanotube structure like iron oxide also contributes to the viability and toxicity to the neurons and inhibitory effect regarding the differentiation of PC12 cells of brain [50]. The excitation in the nerves of CNS involves glutamatergic synaptic transmission pathway, the administration of multi walled carbon nanotubes causes alteration in the glutamatergic transmission and excitatory response of nerve cells in rat hippocampus [51]. In another study illustrating the impact of MWCNTs administration in the rat hippocampus neurons suggested that the MWCNTs inhibits the potassium channels and induces the excitation in the CA1 neurons of rat's hippocampus[52]. To check the influence of functionalized multi walled carbon nanotubes on the genotype, the study was conducted on the p53 gene in heterozygous pregnant mice, to which a dose of functionalized multi-walled carbon nanotubes was administered which induced cell apoptosis and DNA damage resulting in the growth abnormalities in fetus[53].

Cardiovascular Toxicity of Carbon Nanotubes

Exposure to small particulate matter in air can be considered as a risk for development of cardiovascular diseases. Particulate matter like carbon can exert toxic effects on the cardiovascular system by altering the inflammatory systems like fibrinolytic system. This alteration in turn can give rise to endothelial dysfunctioning, pro-coagulant state and other cardiac effects [54, 55]. The medical utility of the CNTs requires their administration into body and they are in direct contact with the blood and blood components, including the cardiovascular system.[56]. Stapleton and associates, exposed experimental rats with MWCNTs at strength of 3 – 5mg for 5 hours. After inhalation the MWCNTs caused the vasodilating responses in the sub-epicardial arterioles of animal. The MWCNTs were translocated from the pulmonary site to the systemic organs after 24 hours post exposure which led to cytotoxicity, pulmonary inflammation and impaired endothelium dependent dilation in the coronary circulation [57].

In another study, hypertensive rats were injected with SWCNTs at a dose of 2.4-2.7 mg/kg of body weight, through intra-tracheal route. This thickened the arterial blood vessels, resulted in edema and leakage of erythrocytes and caused the myofibers of the heart to degenerate without changing the blood serum levels of ICAM-1, TNF and CRF. The serum levels of angiotensin 1 enzyme and endothelin 1 were elevated [58]. Vesterdal demonstrated that when the SWCNTs were administered through intratracheal route at a dosage of 0.5mg kg−1, there was no change in the endothelium derived vasodilatation in the aorta post 2-26 hours of exposure. Exposure to SWCNTs elevated the production of the reactive oxygen species in the endothelium cells as well as acellularly [59]. It was also demonstrated that the aortic and micro vascular cells uptake the CNTs and this results in an elevated levels of the pro-inflammatory cytokines such as IL-8 and chemokine ligand 2 that increased the expression of the adhesion molecules released by these cells. There was also increased production of reactive oxygen species (ROS) in response to exposure with either SWCNTs or MWCNTs at a dose range of 1.5-4.5 μg/mL. The exposure to carbon nanotubes also disrupted the cell structure and exoskeleton and depressed the trans-endothelial electrical resistance. When epithelial and endothelial cell layers were exposed to carbon nanotubes, it results in enhanced cell permeability. It was also found that MWCNTs promoted migration of cells in HMVEC (human micro vascular endothelial cells) [60-62].

MWCNT's have also been evaluated for their potential to cause cardiac ischemia and reperfusion. The cardiac infarct increased in size and ischemia/reperfusion injury was observed in rats following the intra-tracheal administration of 100μg/rat of MWCNTs. Meanwhile, lower doses of 1 to 10μg/rat were not related to causing this type of injury. It was concluded that administration of the MWCNTs increased the cardiac injury and decreased the coronary blood flow by activating the vasoconstrictive mechanisms involving ET-1, thromboxanes and cyclooxygenases [63]. Cao and coworkers have recently published a report demonstrating that when mice were exposed to MWCNTs at 25.6μg/kg/week for 5 weeks through the intra-tracheal administration, the oxidative stress, pulmonary inflammation and the atherosclerotic region of plaque increased. Meanwhile, there were no modified responses on cytokines and isoprostane systemically [64]. In another study, an atherosclerotic model was developed in Male Sprague-Dawley rats weighing 180-200 grams. MWCNTs were inoculated through the intravenous route at a dose of 200μg/kg twice a week for 4 months. This increased the plaque lesion in the aorta from 50% to 66%. Lower doses of 50 or 100μg/kg did not cause the progression of plaque in the aorta of the rats that were made atherosclerotic by feeding them with a high cholesterol diet and injecting them with high doses of vitamin D prior to exposure with MWCNTs. Moreover,

MWCNTs injured the aorta endothelium by disrupting endothelial tight cell junctions and causing endothelial cell death [65].

Nephrotoxicity of Carbon Nanotubes

Kidneys are a vital organ for the human body since they perform essential roles in the maintenance of blood

pressure, acid-base balance, electrolyte homeostasis and the excretion of metabolic wastes. Nanomaterials are able to penetrate the secondary tissues of the body including the kidney. Carbon nanotubes have shown to have some potential toxic effects on kidneys. Carbon nanotubes mostly caused nephrotoxicity through oxidative stress. MWCNTs were evaluated for their toxicity on an *in-vitro* model of human renal cell (engineered HK-2 cells). Two different types of MWCNTs accompanied by seven other types of nanomaterials were found to elevate production of inflammatory mediators like IL8 and IL6 and caused DNA damage. However, the TNF- α and MCP-1 remained unaffected [66].

In order to assess the cytotoxicity and to elucidate its mechanism, human embryonic cell kidney lines (HEK293 cell lines) were also used. After being exposed to SWCNTs (single walled carbon nanotubes) for 48 hours to evaluate toxicity of different parameters such as cell viability, cell cytotoxicity, plasma membrane damage, glutathione level and lipid peroxidation products were recorded. When the cells were subjected to SWCNTs at a concentration of 3-300mg/ml, it resulted in a reduced viability of the cell depending upon the increase in the concentration. Exposure of these cells to SWCNTs at a concentration of 10-100mg/ml caused damage to plasma membrane and also elevated levels of IL-8 and reactive acidic substances like malondialdehyde with depressed intracellular glutathione levels. Thus, exposing the HEK293 cells to SWCNTs concentration dependent cytotoxicity was observed in association with elevated oxidative stress[67]. In case of MWCNTs (multi-walled carbon nanotubes) of size range 60-80nm (MWCNT2) and 90-150nm (MWCNT1), toxicity was observed in the HEK293 embryonic cell line where the MWCNTs of both sizes were responsible for causing high oxidative damage, disrupting the function of plasma membrane (evident by elevated lactate dehydrogenase enzyme) and mitochondria leading to significant cytotoxicity in these cells. In addition MWCNTs also reduced intracellular levels of glutathione, increased IL-8 production and TBARS (Thiobarbituric Acid Reactive Substances). The oxidative stress and cellular toxicity was much higher in the cells exposed to MWCNT2 in comparison to the cells exposed to MWCNT1[68].

The cytotoxic effects of the SWCNTs solubilized in water through sodium dodecyl sulfate were evaluated on epithelial kidney cells of rat known as (NRK-52E). The SDS-SWCNTs at a concentration of 0.125-10μg/mL were subjected to NRK-52E cells resulting in reduction of the cell integrity and elevation of the cytotoxicity. SDS solubilized SWCNTs also resulted in inhibition of the cell growth by arresting the cell cycle at the G0/G1 phase. The proteins involved in the cell cycle such as that of CDK6, CDK2 and pRB (phosphorylated retinoblastoma) were highly depressed by SWCNTs. However, when concentration of SWCNTs was increased, it resulted in cell apoptosis and single cell DNA breakage during growth cycle. Altogether, it was concluded that these SDS solubilized CNTs produce genotoxcity in renal epithelial cells, growth arrest and activate apoptosis on Exposure to the SDS solubilized SWCNTs as the concentration is increased [69]. The effect of the carbon nanotubes on the renal epithelial cell lines was assessed by incubating them with the renal epithelial cell line for a period of 48 hours. At reduced concentrations, the cells that were treated with the carbon nanotubes with reduced trans-epithelial electrical resistance known as TEER. Meanwhile, low concentrations did not affect the ion-based hormone release, cytokine release and levels of lactate dehydrogenase enzyme. TEER is inversely proportional to the CNT concentration evident by the change in the protein expression dependent on the dose. Thus CNTs influence the TEER at low physiological concentrations and do not affect the hormone release, cytokine levels and cytotoxicity at low doses [70].

Hepatotoxicity of Carbon Nanotubes

SWCNTs and MWCNTs induces hepatotoxicity and to less level genotoxicity in the invitro liver model [71]. Oxidized SWCNTs induces the cytotoxic response in the human hepatoma HepG2 cells invitro by the cellular oxidative stress pathway with elevated level of apoptotic hepatocytes [72]. Following IV administration, the comparative hepatotoxic effect of PEGylated and non-PEGylated MWCNTs in mice was studied. The micrographic examination showed that the hepatotoxic effects of PEGylated MWCNTs were less severe as compared to non-PEGylated MWCNTs [73]. Hepatotoxicity induced by MWCNTs is also reduced by surface functionalization like carboxylation

of MWCNTs [74]. The effect of functionalized MWCNTs on the oxidative stress induced apoptotic pathway was observed by various oxidative stress biomarkers like generation of reactive oxygen species in Swiss Webster mice, inflammation and results suggested that the functionalized carbon nanotubes induces hepatotoxicity[75]. The long term dose dependent hepatotoxic response of multi-walled carbon nanotubes was observed in Swiss Albino mice and results suggested the prevalence of hepatotoxic response; tissue apoptosis, cell death etc. [76].

Dermal Toxicity of Carbon Nanotubes

In literature, the dermal toxicity of the carbon nanotubes is very less reported. The reason is that with the passage of time, the development in the field of nanotechnology, the engineering of nanomaterial is modified by the functionalization of nanomaterials like the coating of nanotubes with certain biomolecules as well as the polymers reduced the dermal toxic potential of carbon nanotubes as well[26]. However, mechanism of the dermal toxicity of carbons nanotubes is reported which involves the production of reactive oxygen species, the cytokines; inflammatory mediators results in keratin cytosis [77].

A comparative study of dermal toxicity by unpurified, purified and the carboxylate functionalized multi walled carbon nanotubes in human skin keratinocytes *in vitro* showed that all MWCNTs produces the DNA damage as well as the cellular apoptosis but there is difference in the amplitude of toxicity because the functionalization has the cytoprotective effect to skin cells to certain level[78].

Conclusion

Both SWCNTs and MWCNTs have been associated with organ specific cytotoxicity. Most of research reports pulmonary toxicity caused by CNTs with minimal studies demonstrating skin associated toxicity. The major mechanism of this toxicity has been associated with elevated levels of inflammatory mediators and reactive oxygen species. A lot of studies have been conducted on therapeutic implications of carbon nanotubes however, among them only few have reported potential toxicity of CNTs on major organ systems. This uninvestigated toxicological aspect of CNTs is major hindrance in development of a seemly recognized therapeutic drug carrier and thus, bounds the momentum of research in discipline of CNTs.

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