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Article

Experimental Study of Bio-Security of Functionalized Single-Walled and Multi-Walled Carbon Nanotubes

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Abstract

In this study, the biological effects of functionalized carbon nanotubes (CNTs) were investigated on cell morphology, proliferation, apoptosis, tissue pathology and blood test in vivo & in vitro. The functionalized CNTs had good biocompatibility at lower concentrations, and the functionalized single-walled carbon nanotubes(SWCNTs) perform in the early period in the animal body and multi-walled carbon nanotubes(MWCNTs) mainly in the late. The results show successful functional groups and no change in toxicity in functional samples compared with the primary sample, and there is a safety dosage on the normal cells and tissue. In subsequent studies, antitumoral investigations of modified samples will be evaluated.

Keywords: functionalized carbon nanotubes, bio-security, pathology

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1. Introduction

The materials below nano-scale (0.1-100 nm) have a special nature, so nano-materials have broad application prospects in almost all areas.Carbon nanotubes include of single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). Carbon nanotubes have been attracting increasing attention from chemists and scientists owing to their electronic, mechanical, optical, and chemical characteristics [1-3]. Biomedical applications for CNTs are being investigated actively because of their useful combination of size and physicochemical properties [4-8]. In patients with cancer, MWNTs have potential roles in delivering pharmacologic agents, as diagnostic imaging agents, DNA, silent interfering RNA, oligonucleotides, and proteins to detect or treat cancerous cells [9-11]. The application of functionalized CNTs as new nanovectors for drug delivery was apparent immediately after the first demonstration of the capacity of this material to penetrate into cells. CNTs can be used to deliver their cargoes to cells and organs. In recent years, chemical functionalized of CNTs has become more interesting because it allows modification of the nanotube surface for subsequent alignment. These surface modifications play an important role for application of nanotubes in composite sensors and many other fields. The chemical modifications of CNTs have been well summarized in several published review articles. In order to bind biological macromolecules onto CNTs, the surface is generally required to be oxidated by carboxyl or hydroxyl groups. On the one hand, the functionalized CNTs combine proteins or DNA by covalent binding or a combination of electrostatic interactions, on the other hand, increase its hydrophilic, making it easier to disperse in solution. In this study, with a mixed acid purification, the CNTs surface was rich in hydroxyl and carboxyl functional groups to make it functional. In this study, we investigated the biological effects of functionalized CNTs on normal rat liver, kidney and nerve cells proliferation at the cellular level and normal SD rat heart, liver, kidney structure and function at the animal level, to provide experimental data for the evaluation of CNTs in cell and animal bio-safety and the CNTs using in bio-medical treatment.

2. Materials and Methods

2.1 Test materials and reagents

DMEM and RPMI 1640 medium, fetal bovine

serum(FBS), 0.25% trypsin-EDTA mixture(containing 0.25% trypsin w/v and 0.02% EDTA w/v), and antibiotics solution(containing 1000 U mL^{-1} penicillin and 10 mg mL^{-1} streptomycin) were purchased from Gibco.

2.2. Preparation of test samples

Functionalized SWCNTs and MWCNTs, purified by mixed acid to make it rich in hydroxyl and carboxyl functional groups on the surface, were provided by the College of Mechanical and Engineering, Shanghai Jiao Tong University.

2.3 Toxicity in vitro

2.3.1 Cell culture

Normal rat liver cell BRL-3A, kidney cell NRK, Schwann cell RSC96 were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences(Shanghai, China). BRL-3A cell was maintained in RPMll640 essential medium supplemented with 10% FBS v/v and P/S, and incubated at 37^0C under 5% CO_2. Cells were subcultured every 3 to 4 days. NRK and RSC96 cells were maintained under DMEM medium with the same condition. When the cells were fully adherent, certain concentrations of functionalized CNTs were add to co-cultured with the cells.

2.3.2 Cell proliferation

Logarithmic growth phase of three kinds of cells were seeded in 96 well plates at a density of BRL-3A 3000cells per well, NRK 4000 cells per well and RSC96 2500 cells per well, incubated at 37^0Cand under 5% CO_2 for 24 h. The functionalized CNTs were diluted in the culture medium immediately before addition to each well at the desired final concentrations (10, 50, 100, 150μg mL^{-1}), and the treatment lasted for 24 h, 48 h, 72 h and 96 h. Each concentration was located with three parallel holes, set the control group without CNTs, and set the blank hole. For determining cell viability, the WST-1 assay was used. 10μL of WST-1(at 5mg mL^{-1}, Nanjing KeyGen Biotech CO., Nanjing, China) working solution was added to each well at a final concentration of 500μg mL^{-1}. After the mixture in each well was incubated for 2.5 h, the absorbance was read with a microplate reader (uQuant, Bio-Tek Services, Inc., Richmond, VA) at 450 nm wavelength. The relative cell viability was expressed as a percentage of the control well that was not treated with functionalized CNTs. The inhibition rate was calculated by the formula: cell inhibition rate = [1-absorbance of experimental group/control group absorbance] × 100%.

2.3.3 Cell apoptosis

Logarithmic growth phase of cells were seeded in 6 well plates at a density of 10^5 mL^{-1}, incubated at 37^0C and under 5% CO_2 for 24 h. After treatment with or without functionalized CNTs for 48 h, at the concentration of 100 and 150μg mL^{-1}, cells were harvested by trypsinization and washed once with phosphate-buffered saline (PBS, pH 7.4). After centrifugation, the cells were resuspended by 100 μL Binding Buffer, and then stained with FITC-Annexin V and Propidium Iodide (Invitrogen) for analysis of cell apoptosis. After a reaction under dark condition for 10min, the cells were added 400 μL Binding Buffer, and then analyzed using a flow cytometer(Model BD LSR II, BD Bioscience, San Jose,CA), channel selection FL1 and FL2.

2.4 Toxicity in vivo

40 clean grade SD rats were purchased by the Xinhua Hospital Research Center animal room. When the rats were fed a week in the animal center, they were randomly divided into 3 groups: the control group 16, MWCNT group 12, SWCNT group 12. Then SWCNT and MWCNT groups were divided into four experimental groups (3,7,14,28 days experiment group), with three rats per group. At the 1st,14th,21st and 25th day, the CNTs dispersion were injected into the tail vein, at a dose of 5mg/kg, while two mice of the control group were injected the same dose of saline each time. The CNTs dispersion were blended by ultrasonic processor(KQ-500DE, Shuzhou, China)with 5 min before injection Finally at the 28st day, all the rats were anesthesiaed with ketamine, then taken the heart blood 5 mL, with 1.5 mL for the normal blood test and 3.5 mL for the detection of liver, kidney function and enzymes (blood parameters were obtained from the Xinhua hospital laboratory). After blood collection, we separated the rat heart, liver, kidney and fixed them in 10% formalin, preparing pathological section by the pathology department.

2.5 Statistics

All values are expressed as mean±standard deviation (S.D.). The one-way analysis of variance (one-way ANOVA), followed by Dunnett's multiple comparison post test, was used to verify the significance of a positive response. AP value <0.05 was considered statistically significant. Statistical analysis was performed with SPSS18.0.

3. Results

3.1 Toxicity in vitro

3.1.1 Cell morphology

We observe the cell morphology and take pictures with a digital camera, showed in Fig.1. Compared with the control group, there is no significant difference at the four time points in the low-dose groups (10-100μg mL^{-1}). But the high-dose group (150μg mL^{-1}) shows some differences with the control group. At the 24 h and 48 h points, there is no difference in number and cell morphology, but when

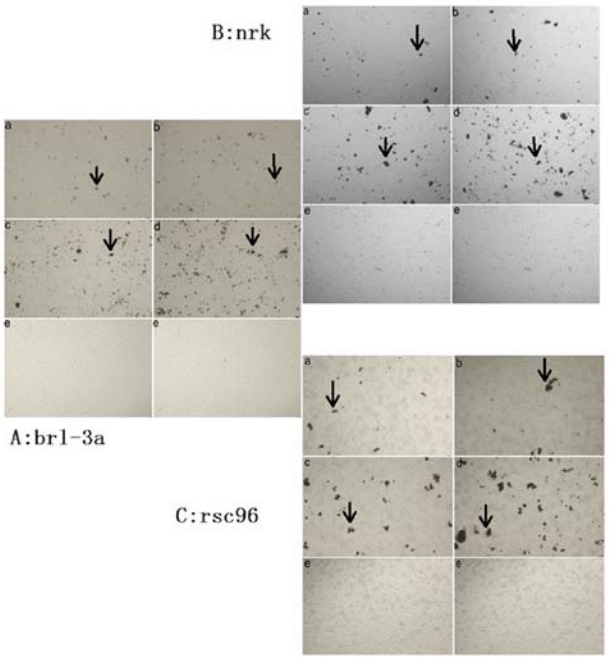

Fig. 1 Functionalized CNTs were co-cultured with three kinds of cell: A and C are MWCNT, B is SWCNT. The a, b, c, d and e respectively shows 10, 50, 100 and 150μg ml^{-1} group and the control group means the reunion of the CNTs.

time comes to the 72 h and 96 h, cells in the experimental group round and shrink, intercellular gap widened, and the number of cell decrease.

3.1.2 Cell proliferation

We measure the absorbance (OD) value and analyze statistically (Fig.2). Similar to the cell morphology result, there is a dose-and-time-dependent phenomenon. The results show that there is no significant difference between the experimental group (dose 10-100μg mL^{-1}) and the control group, but at the dose of 150μg mL^{-1}, the experimental group performs some differences with the control group. At the 72 h point, the inhibition rate is over 50%, and the inhibition rate of three cells is showed in Table 1.

Table 1. The inhibition rate of 150 μg mL^{-1} functionalized CNTs at 72h.

	BRL-3A	NRK	RSC96
SWCNT	61.4%	81.6%	56.8%
MWCNT	64.9%	76.7%	56.8%

3.1.3 Cell apoptosis

Following the cell proliferation observation, we then further determine the effect of functionalized CNTs-induced cell death. As shown in Fig. 3, when we treated three kinds of cells with functionalized CNTs for 72 h, at the concentration of 100 and 150μg mL^{-1}, we find that there is no significant difference between the 100μg mL^{-1} group and the control group, which means that this concentration of the two kinds of functionalized CNTs have no effect on the cell apoptosis. But according to

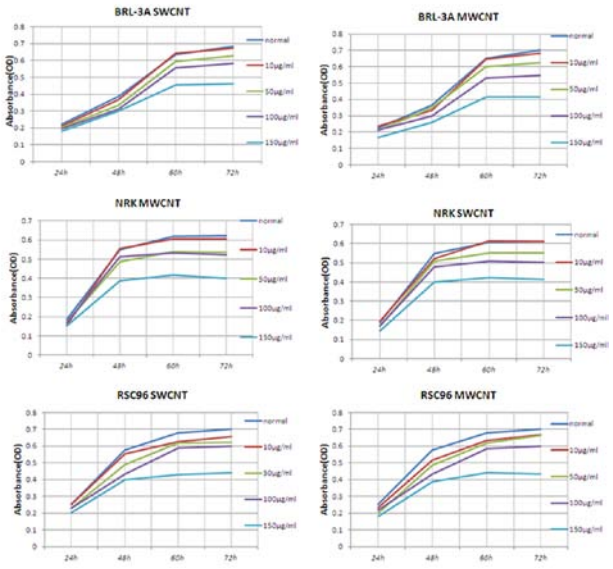

Fig. 2 The effect on cells' proliferation of functionalized CNTs.

the apoptosis data, the concentration of 150μg mL^{-1} CNTs increases the apoptosis rate significantly, by 10 to 15 percentages. The same to say that 150μg mL^{-1} of functionalized CNTs significantly promote cell apoptosis (Tab.2).

Table 2. The effect of cell apoptosis rate of functionalized CNTs.

Item	CON	SWCNT	MWCNT	150μg mL^{-1}
		100μg mL^{-1}	100 μg mL^{-1}	
BRL-3A	9.2%	9.7%	9.6%	19.6%
NRK	6.9%	7.1%	5.8%	22.2%
RSC96	8.5%	9.2%	7.7%	19.6%

3.2 The toxicity in vivo

In heart, We can see focal areas of myocardial atrophy, stained cytoplasm, widened cell gap, infiltration of fat cell is visible in some individual cells gap, changes mainly occurred near the site of the epicardium. In liver, the structural changes are edema and fatty degeneration only in the surrounding areas, the cable structure is clear in central area of liver lobules. There are no blood cells and inflammatory cells, and the morphology of liver cells is normal. In kidney, apocrine secretion increase significantly in the proximal tubules, and the changes focuse in the outer cortex, but renal corpuscles and distal tubules ware normal, with the same to the medullary nephron. In blood, there is no significant change in the number of blood cell and proportion. Among the biochemical parameters, ALT and AST had fluctuations and ultimately returned to normal range. After a slight elevation, BUN and Cr fell to normal of renal function. Three kinds of enzymes have minor changes, but no significant difference with the normal control group (Fig.4, Tab.3).

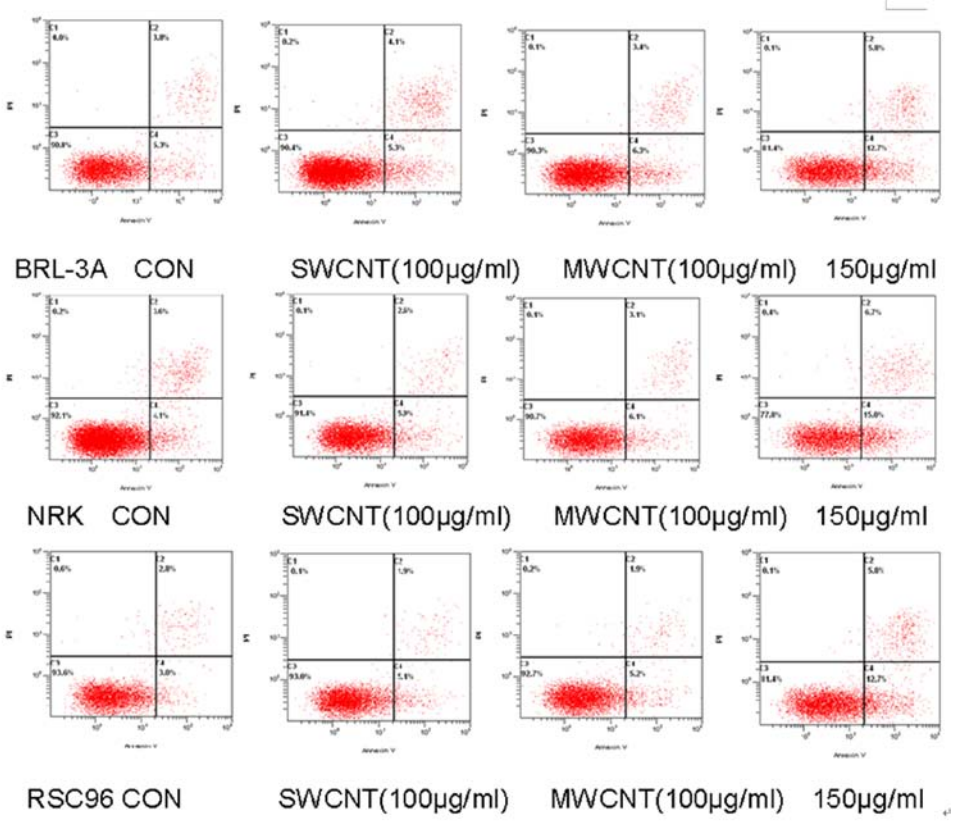

Fig. 3 The effect of functionalized CNTs on cell apoptosis rate.

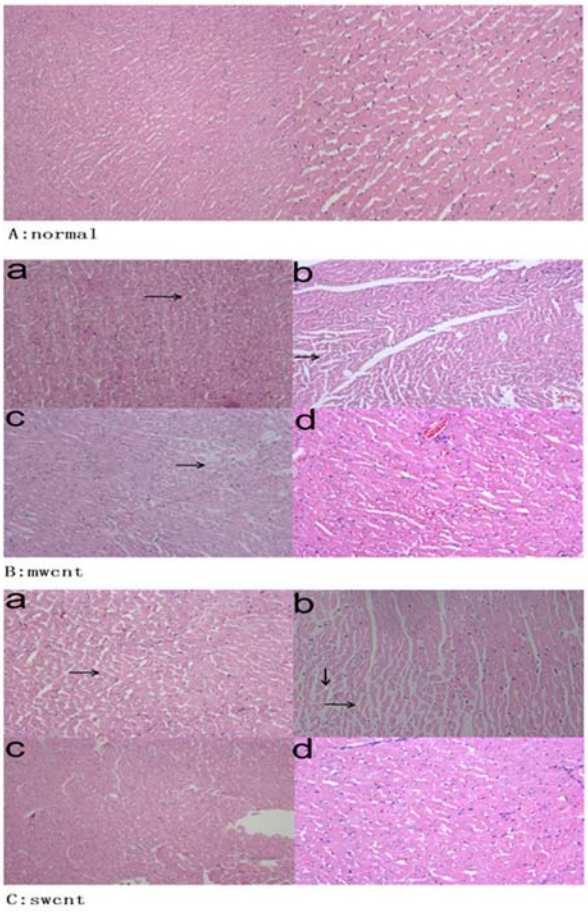

Figure 4A: a, b, c and d respectively for the 3rd,7th,14th and 28st day. A : group of normal rat heart, myocardial cell structural is integrity, cytoplasm lightly stained, tightly packed cells, the gap is small. B: MWCNTs injected group, a significant change was only found in the local area, the wider intercellular. b, c some areas there has been myocardial atrophy, cell morphology became round, deeply stained cytoplasm, the cell gap widened significantly, increased over time, but no inflammatory cell infiltration. →myocardial contraction. d myocardial cell morphology and structure have become normal myocardium. C: SWCNTs injected group, a, b appeared in some areas of myocardial atrophy, cell morphology became round, deeply stained cytoplasm, the cell gap was widened, and a fat cell infiltration, increased with time, but no infiltration of inflammatory cells into cardiac muscle, →atrophy, ↓ fat cell infiltration. c, d myocardial cell morphology and structure have become normal myocardium.

4. Discussion

Generally speaking, the CNT size, degree of aggregation and surface chemical modification is the major factor that impacts the cytotoxicity. Some study showed that SWCNTs had more toxic than MWCNTs [12], mainly because that the diameter of SWCNTs is smaller than that of the MWCNTs, which is more easily to go into cells. In this study, two kinds of functionalized CNTs were co-cultured with three normal cells to watch the cell morphology. We found that the 150μg mL^{-1} group appeared in significant changes in cell morphology, which may be related to the degree of aggregation. In the preparation of CNT suspension, we found that it is difficult to disperse the material. It was easy to

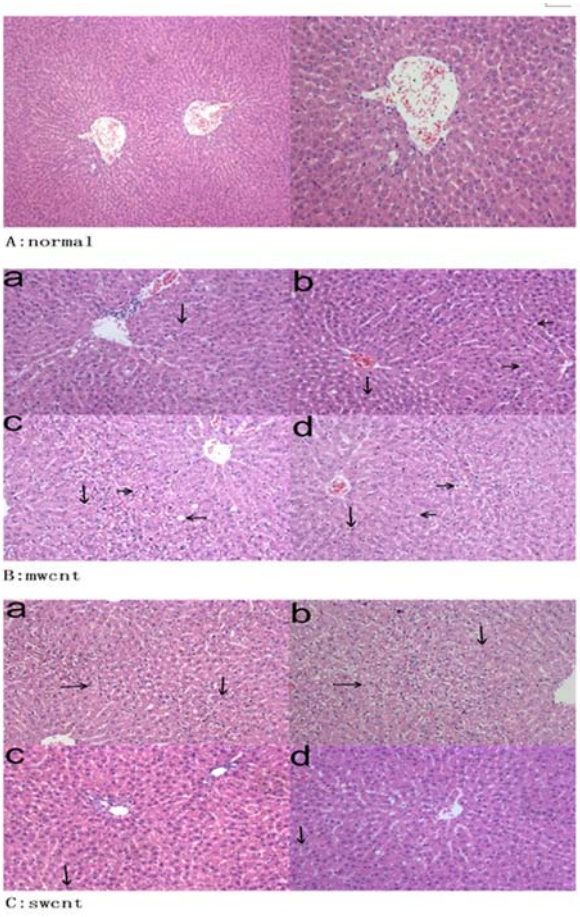

Figure 4B: a, b, c and d respectively for the 3rd,7th,14th and 28st day. A: group of normal rat liver, hepatic lobule structure complete, tightly packed, liver cord structure is clear, heparin is no inflammatory cells and blood cells. B: MWCNTs injected group, a significant change was only found that a partial deposition of CNTs or phagocyte. b, c part of the region appeared disordered structure, cell edema and fatty degeneration, the progress of leaflet edge to the center, the deposition of CNTs or phagocyte, increased with time, but no inflammatory cell infiltration; d lobular structure has clearly improved over the previous two groups, but there is still adaptive changes and CNTs deposition, →cell edema, ←fatty degeneration, ↓CNTs deposition. C: SWVNTs injected group, a, b some areas there has changes in the structure of hepatic lobule, the progress from the edge to the center of lobules, changes of cell edema and deposition of CNTs or phagocyte, increased with time, but no inflammatory cell infiltration, →cell edema, ←fatty degeneration, ↓CNTs deposition. c, d liver cell morphology and structure have become normal hepatic lobule, CNTs deposition is still there, ↓CNTs deposition

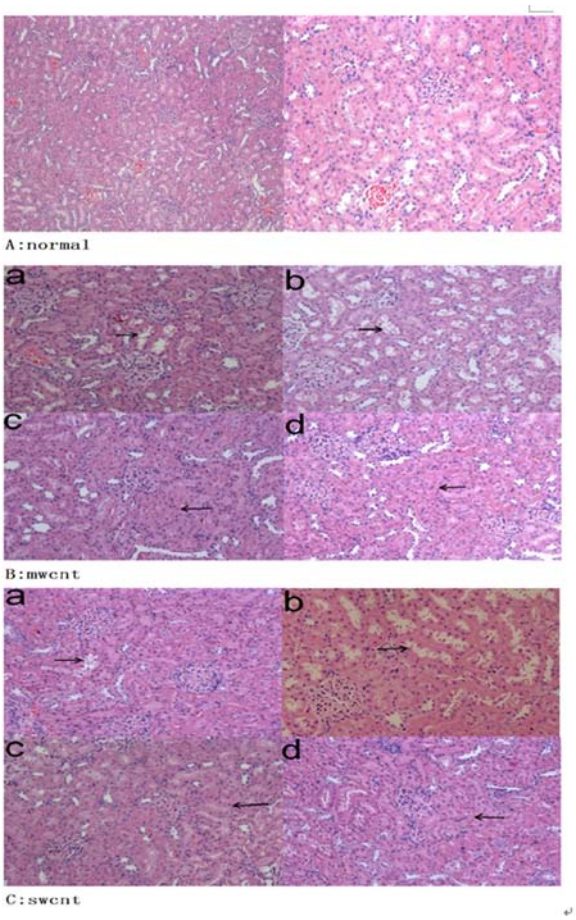

Fig. 4C: a, b, c and d respectively for the 3rd,7th,14th and 28st day. A: group of normal rat kidney, renal unit structural integrity, deeply stained proximal tubule, distal tubule lightly stained. B: MWCNTs injected group, a, b in the proximal tubules of apocrine secretion increased, increased with time, →apocrine secretion. c, d proximal tubule of apocrine secretion is normal, but some areas there has been tightly packed proximal tubule phenomenon, ←packed proximal tubule. C: SWCNTs injected group, a, b in proximal tubule apocrine secretion increased, reduced with time, →apocrine secretion. c, d proximal tubules of apocrine secretion is normal, but some areas there has been the phenomenon of the proximal tubules tightly packed, reduced with time.

agglomerate, so we had to use ultrasound to disperse the material prior. Wick et al [13] examined the different degree of aggregation of SWCNTs on human MSTO-211H cell toxicity and found that a large gathering can induce greater hardness and size, and thus have greater toxicity. The difference with previous reports [14] is that between the SWCNTs and MWCNTs we didn't find differences in the impact of cell morphology.

MTT assay is a commonly used cell number and viability detection methods, but studies have shown, the formazan particle, generated in the MTT procession, was easily absorbed to CNTs surface, and transmission electron microscopy showed that the formazan-CNTs combination was not easy to be dissolved by DMSO, which decreased the absorption caused false-positive results [15].But, in the WST-1 experiment, such phenomenon wound not appear. WST-1 is a more sensitive, more stable compounds, similar to MTT, the electron coupling reagent in the presence of, can be reduced to water-soluble formazan by some dehydrogenase in the mitochondria, which can be dissolved in tissue culture medium. The amount proportional depended on the amount of cells. WST-1 results showed that the functionalized CNTs have a certain concentration range of security on the three kinds of normal rat cell, beyond this range would show cytotoxicity. And they showed greater toxicity in the kidney cells.

The two kinds of materials caused different pathological changes. In the liver, SWCNTs caused cell edema, and the changes induced by MWCNTs were edema and part of the fatty degeneration, occurred in

Table 3A. The changes of blood parameters by MWCNTs

Item	Normal	1 day	3 day	7 day	14 day	28 day
WBC(10^9 L^{-1})	3.76±1.18	3.91±1.25	5.28±2.36	4.94±1.24	2.6±1.05	3.09±1.89
RBC(10^{12} L^{-1})	8.12±0.76	8.18±0.74	7.58±0.67	7.86±0.71	7.33±0.53	8.43±0.84
HB(g L^{-1})	147±15	154±13	183±27	205±26	146±10	165±18
BPC(10^9 L^{-1})	1132±111	1427±123	1675±180	1361±141	1131±123	1298±195
N(%)	16.7±2.4	29.7±4.1	25.9±3.8	17.4±2.7	13.1±2.2	18.7±2.6
L(%)	79.3±5.5	54.2±4.5	66.5±4.8	74.9±5.1	78.8±5.8	69.7±5.1
ALT(u L^{-1})	28±6.49	37±8.41	40±7.54	47±8.19	44±7.08	28±6.56
AST(u L^{-1})	91±26.54	89±26.75	101±29.14	117±32.74	87±25.47	78±21.89
TP(g L^{-1})	61±4.6	72.1±5.1	62.9±4.9	65.8±5.4	65.2±5.8	60.8±4.2
ALB(g L^{-1})	40.3±1.96	45±2.52	40.9±1.95	42.3±2.02	41.9±1.98	42.2±1.67
BUN(mmol L^{-1})	5.79±1.22	7.99±2.6	9.25±2.39	9.97±1.98	8.12±2.17	7.15±2.25
CR(μmol L^{-1})	31±8.3	39±9.6	46±13.7	38±10.7	36±5.7	30±6.7
CK-MB(u L^{-1})	323±21.4	526±94.6	649±132.7	540±90.3	426±75.9	386±50.1
HBDH(u L^{-1})	207±24.5	291±43.6	349±49.5	280±35.7	274±30.5	242±26.3
LDH(u L^{-1})	278±21.8	486±70.6	417±57.6	365±50.3	263±27.7	225±20.4

Table 3B. The changes of blood parameters by SWCNTs

Item	Normal	1 day	3 day	7 day	14 day	28 day
WBC(10^9 L^{-1})	3.76±1.18	4.95±2.28	5.51±1.96	5.55±1.96	5.42±2.39	3.57±2.43
RBC(10^{12} L^{-1})	8.12±0.76	8.27±0.78	7.32±0.65	7.39±0.67	8.11±0.75	8.71±0.84
HB(g L^{-1})	147±15	152±17	142±14	138±12	149±18	175±21
BPC(10^9 L^{-1})	1132±111	1345±142	1461±183	1361±194	1040±107	1289±167
N(%)	16.7±2.4	22.6±2.9	27.7±3.6	12.6±2.2	10.1±2.1	16.3±1.6
L(%)	79.3±5.5	67.7±5.2	65.2±4.9	81.2±5.7	80.6±5.6	81.2±5.8
ALT(u L^{-1})	28±6.49	38±6.91	36±6.84	25±6.79	23±5.67	29±7.89
AST(u L^{-1})	91±26.54	107±27	114±30	88±22	83±21	95±30
TP(g L^{-1})	61±4.63	61.3±467	64.5±4.72	66.3±4.89	70.1±5.33	61.1±4.64
ALB(g L^{-1})	40.3±1.96	38.9±1.92	41.4±2.01	43±2.34	46.7±2.47	34.6±1.75
BUN(mmol L^{-1})	5.79±1.22	6.55±1.45	7.11±1.56	6.45±1.59	5.77±1.35	5.42±1.27
CR(μmol L^{-1})	31±8.3	45±10.3	39±8.5	33±8.4	36±9.1	28±7.5
CK-MB(u L^{-1})	323±21.4	532±90.4	431±56.3	287±40.7	293±45.2	359±36.8
HBDH(u L^{-1})	207±24.5	329±50.5	328±58.3	259±37.4	199±32.4	198±16.5
LDH(u L^{-1})	278±21.8	482±62.6	360±53.6	288±30.5	244±26.6	272±14.6

the lobular edge position. In the three tissues, MWCNTs induced changes mainly in the middle and late stage, while SWCNTs mainly in the early period, which seemed to say that the MWCNTs showed more pathological efficacy. Accompanied by organizational changes, the relevant blood parameters had some volatility, but there was no difference between the two kinds of CNTs. Every experimental group didn't find significant inflammatory cell infiltration. After intravenous injection of CNTs, each experimental group of mice didn't perform any serious pathological changes, such as apoptosis, necrosis. The result demonstrated that the tissue had a certain self-repair capacity to treat the adaptive changes in the body.

Whether surface modification of CNTs shows some cytotoxic effect, the current experiment studies still have some controversy. Bottini et al [16] compared the original with the carboxylic MWCNTs by nitric acid, found that

oxidation of the CNTs showed higher toxicity. But Kam et al [17] reported that nitric acid oxidized SWCNTs co-cultured with human HL60 promyelocytic leukemia cells at the concentration of 50μg mL^{-1}, after 24 and 48 h there showed no cell death, indicating that the functionalized SWCNTs had no toxic effect on HL60 cells. Sayes et al [18] found that acid-modified and isophthalic acid-modified SWCNTs showed smaller toxicity than the original material on human dermal fibroblasts, pointed that cell toxicity depended on functional density, the higher functional density, the smaller cytotoxicity. The original SWCNTs were more toxic than the SWCNTs removed metal impurities [19]. In summary, modifying the surface of the CNTs will show better biocompatibility [20]. Rare earth elements have the feature of low electronegative and large activity, which can be used as surfactants and shallow infiltration of elements [21], because of the typical electronic structure (- - - 4f0-14). If we use the rare earth elements to function the surface of CNTs, they can clean the surface of the CNTs, and reduce the surface energy, to be better biocompatibility and more easily dispersed.

The functionalized MWCNTs and SWCNTs have good biocompatibility at lower concentrations, the functionalized SWCNTs perform in the early period in the animal body and MWCNTs mainly in the late, which show a more serious toxicity. Oxidative stress is one of the mechanisms leading to cell toxicity of CNTs, but different chemical surface properties of CNTs may have a different mechanism on cell viability, the mechanism needs further study, and its preparation process also needs further study and improvement. Cells and tissues have the capacity of adaptability and self-repair mechanisms to balance the damage caused by functionalized CNTs, the activity of cells and tissues gradually restored with time, speculating that the removal of cells ROS is one of the reasons, but the specific clearance mechanism has yet needs future study.

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