

Single Wall Carbon Nanotube Induced Inflammation in Cruor-Fibrinolysis System*

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Abstract

Objective To study single wall carbon nanotubes (SWCNT) and its role in inducing inflammatory cytokines in the cruor-fibrinolysis system of rat.

Methods Twenty one Wistar rats were divided into four groups: 1) control; 2) low-dose SWCNT (0.15 mg/kg BW); 3) medium-dose SWCNT (0.75 mg/kg BW); 4) high-dose SWCNT (1.5 mg/kg BW). Intratracheal instillation of SWCNT suspensions was administered to rats once per day for 21 days. In order to assess the exposure effect of SWCNT to the rats, activity of Inflammatory cytokine was measured and markers of cruor-fibrinolysis system were studied via ELSIA. Also, change in clotting time was recorded and histopathology was studied.

Results IL-6 and IL-8 concentrations of rats exposed to SWCNT were significantly higher than those in controls ($P<0.05$). The activity of inflammatory cytokines and histopathological change indicated that oxidative damage occurred. Change in clotting time in rats exposed to SWCNT decreased compared with controls. Meanwhile, t-PA (tissue-tupee plasminogen activator) and AT-III (antithrombin-III) levels in rats exposed to particulates increased or decreased significantly compared with controls ($P<0.05$). A similar trend was observed for D-dimer (D2D) levels, indicating that SWCNT can impact the cruor-fibrinolysis system of rat.

Conclusion The results from our study suggest that an increased procoagulant activity and reduced fibrinolytic activity in rats exposed to SWCNT can cause pulmonary oxidative stress and inflammation, due to the release of pro-thrombotic and inflammatory cytokines into the blood circulation of rat.

Key words: Carbon nanotube; Inflammatory factor; Cruor-fibrinolysis system; Cardiovascular system

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INTRODUCTION

Various epidemiological and clinical studies have explored how do inhaled airborne particulates correlate with the incidence and mortality of cardiovascular disease^[1-5]. Findings from nano-toxicology studies have shown that ultrafine particles and nanoparticles presented in particulate matter, can readily penetrate and

cross the pulmonary epithelium of the lung-blood barrier due to their particle size, charge, and chemical composition^[6-10]. And such translocations has been observed in animals and it was revealed that exposure to nanomaterials may affect the cardiovascular system^[8,10-11].

As a typical nanomaterial, carbon nanotubes (CNTs) have many potential applications, such as being used in the production of high-strength

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materials, reinforced rods, quantum wires, mechanical memory materials, and also in microfabrication of conjugated polymers and extraordinary electronic, light-emitting, and catalytic properties^[12]. And single wall carbon nanotube (SWCNT) has been included in the list of representative manufactured nanomaterials for testing by the Organization for Economic Co-operation and Development^[13], and the toxicological assessment of SWCNT must be therefore conducted. The toxicity of carbon nanotubes has been extensively studied^[14-15], and *in vitro*^[16] and *in vivo*^[17] studies have confirmed that exposure to carbon nanotubes can cause oxidative damage and accumulation of reactive oxygen species (ROS). However, little is known currently about the toxicity of nanomaterial to the cardiovascular system and its pathogenesis.

Inhaled pollutants can cause pulmonary oxidative stress/inflammation and are subsequently responsible for cardiovascular toxicity^[18-19]. The inflammation plays a substantial role in atherogenic progression, causing alterations in endothelial function and potentially mediating acute plaque rupture. Some *in vitro* studies on human bronchial epithelial cells showed that particulate matter exposure can lead to release of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor- α (TNF- α)^[20-21]. Activation of transcription factors and an increased intra-airway transcription of IL-8 lead to an increase in cytokine production, promoting airway inflammation^[22-24].

Previous *in vitro* and *in vivo* studies show that particulate matter induces pro-thrombotic effects^[25-26]. Exposure of rodents to particulate matter results in bronchoalveolar lavage (BAL) fluid proteolysis, platelet function abnormalities and hemostatic alterations that culminate in intravascular thrombosis^[27-28].

It is well known that cytokines such as TNF- α , IL-1, IL-6, and IL-8 can promote the activation of the coagulation system. However, little is known about impact of inflammatory factors induced by nanomaterials on the cruor-fibrinolysis system and it was for this reason, the present study was designed and conducted.

MATERIALS AND METHODS

SWCNT and Suspension Preparation

SWCNT was from NanoLab, Inc. (Waltham, MA, USA). A single tube with a diameter of 0.8-1.2 nm and a length of 100-1000 nm was used. The particles were observed under scanning and transmission electron microscopes. For scanning electron microscope (SEM) analysis, the particles were dried, deposited onto dedicated stubs, and observed under a Tescan SEM VEGA TS 5136 XMEquipped with a SEDAX GENESIS 4000 XMS micro analyser. For transmission electron microscope (TEM) analysis, samples were prepared by suspending particles in appropriate dilutions in 0.01% Tween 20 in distilled water. After being sonicated and vortexed (30 min), drops of about 10 μ L of suspensions were pipetted onto Formvar[®]-coated 200 mesh copper grids. Water was gently blotted, and once dried, grids were directly inserted into a Jeol JEM-100CX transmission electron microscope equipped with a CCD camera. The Raman spectra of the functional group of SWCNTs (RM200, Renishaw, England) is shown in Figure 1C. As shown in Table 1, the chemical composition was 99.16% C, 0.584% O, and 0.249% Si. Carbon nanoparticle suspensions were prepared in normal saline by vortexing three times for 5 s followed by sonication for 1 min in an ultrasonic bath just before intratracheal instillation was administered each time to rats and this procedure was repeated three times.

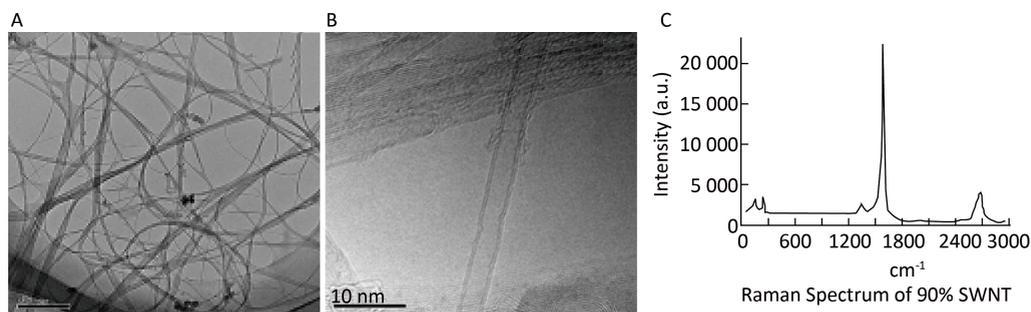


Figure 1. Superficial character of SWCNTs: A: SEM; B: HRTEM; C: Raman Spectrum.

Table 1. Quantification Results of SWCNTs (Correction Method: Thickness)

Element	Weight (%)	Atomic (%)	Uncertainty (%)	Detector Correction	k-Factor	Absorption Correction
C (K)	99.166	99.453	1.455	0.438	2.489	0.974
O (K)	0.584	0.439	0.139	0.495	2.059	0.883
Si (K)	0.249	0.106	0.038	0.978	1.000	0.996
Fe (K)	0.000	0.000	100.000	0.997	1.477	0.999

Animal Treatment

Twenty one healthy male Wistar rats (8 weeks of age, weighing 180-220 g) were obtained from the Academy of Military Medical Sciences (Beijing, China). All procedures concerning the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Academy. Rats were divided randomly into four groups: 1) control; 2) low-dose SWCNT; 3) medium-dose SWCNT; 4) high-dose SWCNT. Then, the rats were anaesthetized with ether, and exposed to the SWCNT suspension via intratracheal instillation at a dose of 0.15 mg/kg body weight (BW), 0.75 mg/kg BW or 1.5 mg/kg BW respectively. The controls were treated with 7.5 mg/kg BW of normal saline. The intratracheal instillation was administered once per day for 21 days. Following the exposure period, the animals were anesthetized with hydral and blood was collected from the abdominal aorta of the rats for biochemistry.

Study on Inflammatory Markers and Function of Cruor-fibrinolysis System in Peripheral Blood

The activity of inflammatory markers (IL-6, IL-8, and TNF- α) and the cruor-fibrinolysis system markers [tissue plasminogen activator (t-PA), D2D, antithrombin-III (AT-III), endothelin-1 (ET-1), nitrogen oxide (NO), and von Willebrand factor (vWF)] were determined using ELISA kits (R & D Systems, Minneapolis, MN, USA), and results were read using an ELISA Reader (Thermo MK3, USA).

Prothrombin time (PT), fibrinogen time (FIB), thrombin time (TT), and activated partial thromboplastin time (APTT) were measured by BD-Compact automated blood coagulation analyzer (BD, Franklin Lakes, NJ, USA). We used in our present study tail clippings to measure bleeding time (BT) and coagulation time (CT).

Histopathological Evaluation

The middle part of the left lung of the rats exposed to SWCNT was immediately fixed in Bouin's solution and processed according to standard

histological techniques. After being preserved for 24 h in the fixative, the fragments were rinsed in distilled water, dehydrated in an ethanol series from 70% to 100% and embedded in Bio-plast tissue embedding medium. For the controls, serial sections were cut by a rotary microtome, mounted on slides and routinely stained with Mayers Haemalaun and alcoholic eosin. Histological samples were qualitatively screened by using a Zeiss Axioplan 40 light microscope and images were taken with a Zeiss AxioCam MRc5 digital camera interfaced with the Axiovision Real 4.6 software.

Statistical Analyses

All statistical analyses were performed using SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA) and the data are represented as mean \pm standard deviation (SD). A one-way analysis of variance (ANOVA) and Bartlett's test were calculated for each value. If the F-statistic from the ANOVA was significant, Dunnett's test was then used to compare the mean from control group to each of groups exposed to the particulates and the differences in the means were considered significant if $P < 0.05$.

RESULTS

Change in Body Weight of Rats Exposure to SWCNT

As shown in Figure 2, the body weight of rats decreased when the dose of SWCNT increased compared with controls and the increase rate of body weight slowed 7 days after the exposure to the particulate, suggesting that SWCNT may have harmful effects on health of rats.

Level of Inflammatory Markers in Rats Exposed to SWCNT

Figure 3 shows the serum levels of proinflammatory cytokines measured as indicators of inflammation in particulate-exposed rats. IL-6 levels in the medium and high dose groups were significantly higher than those in control group ($P < 0.05$). A similar trend was observed for IL-8 levels

($P>0.05$). However, TNF- α concentrations were not different compared with controls.

Histopathological Evaluation

The lung tissue images of the rats indicate pulmonary exposure to nanoparticles produced a persistent and progressive inflammatory lung response

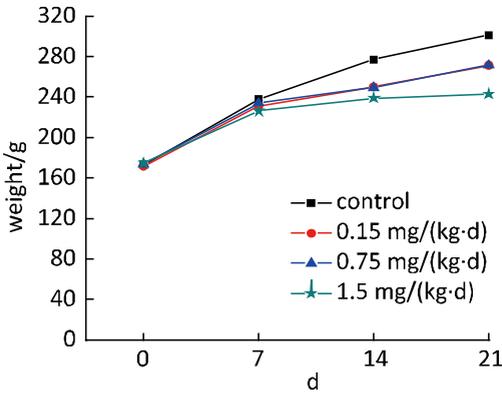


Figure 2. Changes in body weight of rats induced by different concentrations of SWCNT within 21 d.

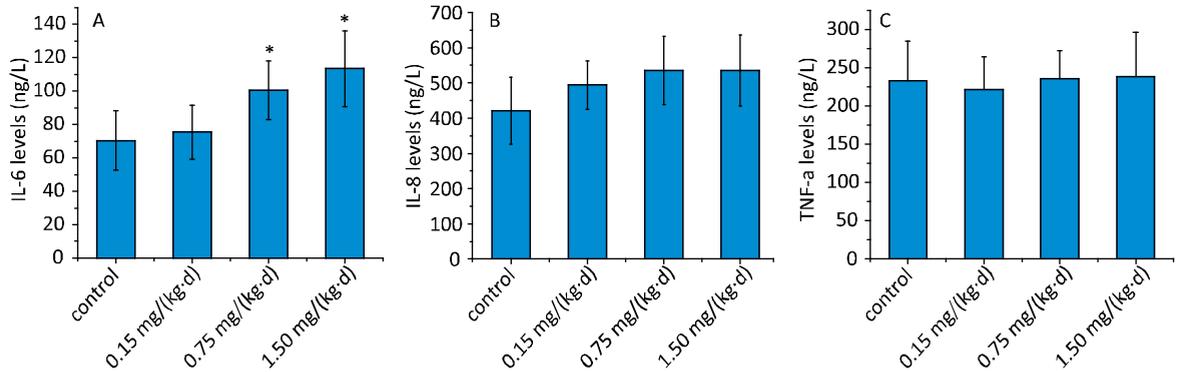


Figure 3. Serum inflammatory markers induced by different concentrations of SWCNT within 21 days: A) Effect of SWCNT on IL-6 contents in serum; B) Effect of SWCNT on IL-8 contents in serum; C) Effect of SWCNT on TNF- α contents in serum. Data are represented as means \pm S.D.. * Statistical significant differences were determined by a one-way ANOVA ($n=6$; $P<0.05$).

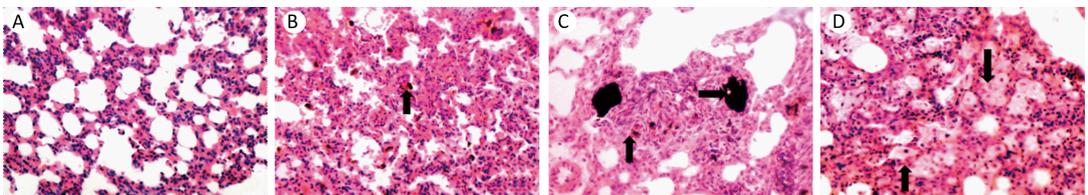


Figure 4. Light micrographs of lung tissue of rats exposed to SWCNT and euthanized 21 d after the single treatment: (A) Control; (B) 0.15 mg/kg per day; (C) 0.75 mg/kg per day; (D) 1.5 mg/kg per day. Particles were scattered in alveoli and granulomas contained black particles. The aggregate of inflammation cells (lymphocytes) around the area surrounded by quartz particle-containing (arrow). Magnifications varied from 3.3×10 to 3.3×40 .

(Figure 4). We also observed blood clots and secondary thrombosis in their pulmonary arteries, inflammatory cell infiltration around alveoli, and local hemorrhages in the alveoli and interstitial tissue. Lung tissue thickening, as a prelude to fibrosis development, was also evident (Figure 4C and 4D). However, these effects were absent in controls (Figure 4A). The lung tissue of the rats exposed to SWCNT showed mild to moderate alveolar and interstitial inflammation, with inflammation predominately inside the edema area, while no inflammation was observed in alveolar tissue in controls (Figure 4A).

SWCNT Induced Changes in Blood Coagulation Function

The changes in PT, APTT, TT, and FIB induced by SWCNT in sera of rats were measured as indicators of blood coagulation (Table 2). In rats exposed to 0.75 and 1.5 mg/kg per day of SWCNT, PT, APTT, and FIB were significantly different compared with controls ($P<0.05$).

Table 2. Effect of SWCNT on PT, APTT, TT, and FIB in Rats

Group (per day)	N	PT (s)	APTT (s)	TT (s)	FIB (g/L)
Control	6	9.16±0.35	18.81±0.86	54.18±5.00	1.57±0.29
0.15 mg/kg	6	9.00±0.29	15.28±1.28	55.11±6.70	1.54±0.38
0.75 mg/kg	6	7.93±0.19 ^a	14.25±0.91 ^a	50.46±4.00	1.73±0.16 ^a
1.5 mg/kg	6	7.68±0.10 ^a	14.20±0.50 ^a	55.50±6.90	1.82±0.13 ^a

Note. ^a $P < 0.05$ compared with control.

Effect of SWCNT on bleeding time and coagulation time in rats are shown in Figure 5. The bleeding time in 0.75 and 1.5 mg/kg per day groups

significantly decreased compared with the controls ($P < 0.05$). Moreover, a similar trend was observed for the coagulation times in rats exposed to SWCNT.

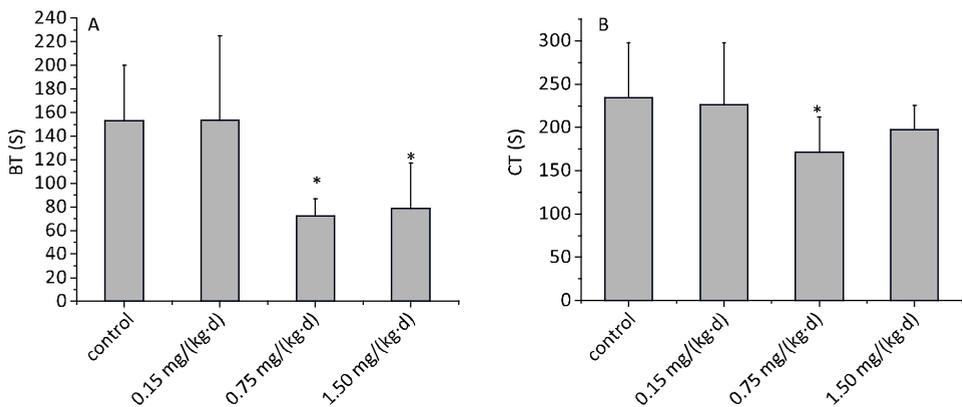


Figure 5. SWCNT induced changes in blood coagulation function: A) Effect of SWCNT on bleeding time in rats; B) Effect of SWCNT on coagulation time in rats. Data are represented as means±S.D.. *Statistical significant differences were determined by a one-way ANOVA ($n=6$; $P < 0.05$).

SWCNT Induced Changes in Anti-coagulation and Fibrinolysis Function

The levels of t-PA, D-D, AT-III, ET-1, NO, and vWF in sera of rats were determined as indicators of blood fibrinolysis following nanoparticulate exposure (Figure 6). t-PA levels in 0.75 mg and 1.5 mg/(kg-d) treated rats decreased significantly compared with controls ($P < 0.05$). Interestingly, the levels of AT-III in rats exposed to the particulates were significantly higher than those in control group ($P < 0.05$) and a similar trend was observed for D2D levels ($P > 0.05$). However, the concentration of ET-1, NO, and vWF in rats exposed to SWCNT were not significantly different compared with controls

DISCUSSION

The pro-inflammatory cytokines (IL-6, IL-8, TNF- α) play an important role in regulating immunity.

The levels of IL-6 in 0.75 and 1.5 mg/kg per day of SWCNT treated rats were significantly higher than those in the control rat ($P < 0.05$), similar to what was observed for IL-8. The results revealed that SWCNT induce an inflammatory response in the peripheral blood of rats and release of IL-6 as a result of particulate induced inflammation targets transcription of procoagulant proteins and decreases transcription of anticoagulant proteins^[29]. Amongst its many activities, IL-6 plays a pivotal role in hemostasis. IL-6 induces a prothrombic state by increasing the expression of tissue factor, fibrinogen, and factor VIII that increases the risk of both venous and arterial thrombosis^[30]. In our study, the results showed that TNF- α concentrations were not different compared with controls and we believe that whether the generation of TNF- α is a direct result of pulmonary exposure to SWCNT or not needs further study.

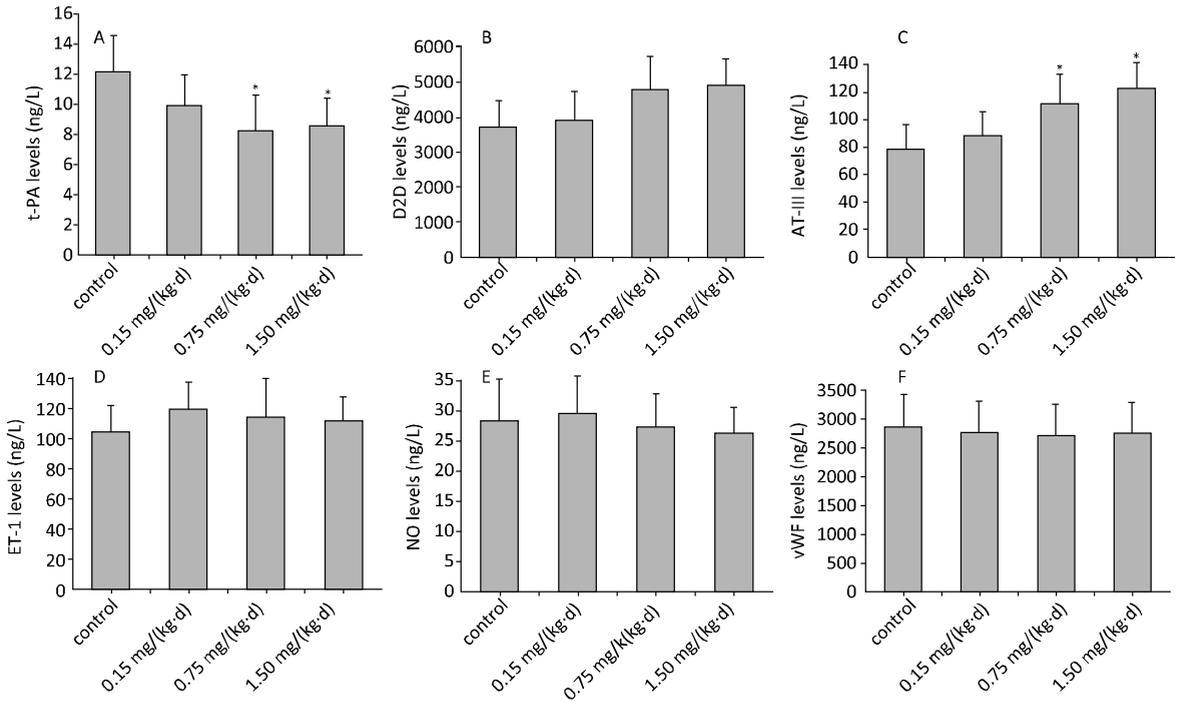


Figure 6. Serum fibrinolysis markers induced by different concentrations of SWCNT within 21 d. A) Effect of SWCNT on t-PA contents in serum; B) Effect of SWCNT on D2D contents in serum; C) Effect of SWCNT on AT-III contents in serum; D) Effect of SWCNT on ET-1 contents in serum; E) Effect of SWCNT on NO contents in serum; F) Effect of SWCNT on vWF contents in serum. Data are represented as means \pm S.D.. * Statistical significant differences were determined by a one-way ANOVA ($n=6$; $P<0.05$).

Histopathological study of lung tissue of rats revealed that pulmonary exposure to SWCNT induces persistent and progressive inflammatory responses in the lung. Injuries to blood vessels activate the body's hemostatic mechanism to repair the damage and avoid loss of blood. This results in accumulation of platelets and fibrin, forming a blood clot or thrombus.

For many years, coagulation defects were analyzed by measuring only clotting and bleeding times. Changes in bleeding time and clotting time can be an indicator of a hemostatic problem, which could be caused by vascular abnormalities, or other factors. The bleeding and clotting times in exposed groups in the present study decreased more than those in control group, indicating that SWCNT had impacted the rat coagulation system. However, both methods were antiquated for such purposes because current methods, such as the time of fibrin formation in recalcified plasma (PT and APTT), are much more sensitive and specific. In 0.75 and 1.5 mg/kg per day groups, PT and APTT significantly decreased compared with controls suggesting that SWCNTs affect both intrinsic and extrinsic coagulation

processes. A simultaneous decrease of PT and APTT reflects defects in a common pathway, and are associated with elevated activity of factor VII with other factors of the intrinsic pathway, such as factors VIII, IX, and XI, or prekallikrein. And there is an important correlation between the number of platelets and the clotting time. It is because platelets are sources of indispensable phospholipids that contribute to the activation of the coagulation factors, which activate the conversion of prothrombin to thrombin, that activates in turn the fibrinogen which stimulates thrombocyte aggregation. Compared with the controls, the levels of fibrinogen and clotting time in exposure groups increased or decreased significantly ($P<0.05$), showing that the coagulation system of rats was activated.

Most of these variables in the cruor-fibrinolysis system can be readily assayed via ELISA. Tissue plasminogen activator (t-PA) is the main fibrinolytic stimulator. Fibrin degrades into soluble fibrin degradation products, including D2D. The primary inhibitor of the fibrinolytic process is the plasminogen activator inhibitor type 1 (PAI-1), which inhibits plasminogen activation by binding with t-PA

to form the PAI/t-PA complexes. Therefore, impaired fibrinolytic function may be reflected in low levels of blood t-PA activity. Reduced plasmin generation leads to suppression of fibrinolytic activity, thus favoring fibrin persistence and thrombosis.

D2Ds are degradation products of cross-linked fibrin. The increase in D2D values in rats exposed to SWCNT in our study showed that there were frequent cross-linked fibrin degradation process. AT-III is one of the most important anticoagulants in whole blood and it is responsible for the inactivation of 50%-60% of thrombin and factor VIIa, IXa, Xa, and XIa in order to prevent blood clotting. AT-III is also a potential lysosomal protease inhibitors, which could reduce the inflammatory response^[31-32]. In this study, the changes of the FIB, APTT, PT, TT, and D2D values in rats exposed to SWCNT indicate that the balance between coagulant and anti-coagulant was disrupted. However, the increase in AT-III value in rats may be related with its anti-inflammatory mechanism action, which needs further study in combination with other parameters such as thrombin, FIX, Fxa, and fibrinolysin.

In our study, the levels of inflammatory cytokine and the results of histopathological evaluation in lung of rats exposed to SWCNT indicated that inflammatory reaction had occurred and procoagulant activity increased and fibrinolysis capacity decreased. The inflammation induced by oxidative stress resulted in enhanced cruor-fibrinolysis system, leading, in turn, the pro-thrombotic effects and the promotion of development of ischemic heart disease.

In sum, the findings from our present study suggested that CNTs could lead to inflammation and oxidative stress when penetrating the alveolar epithelium in rats. Referring to the Nemmar et al., 2003a, b; Chambers 2012^[27-28,33], we therefore hypothesized that the inflammatory cytokines induced by nanomaterial in lung tissue of rats could enhance the inflammatory response in the peripheral blood, resulting in the increase of function of cruor-fibrinolysis system. And under such a situation, inflammatory factors acted as the signal transduction as well as the effect or molecules might play important roles in regulating coagulation, anti-coagulation and fibrinolysis. The mechanism of this process would be our next research focus.

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