Respiratory exposure to single-walled carbon nanotubes induced

changes in vascular homeostasis and the expression of

peripheral blood related genes in a rat model

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Abstract

Epidemiological studies have demonstrated that nanometre particles in polluted air can increase the risk of CVD, which is dangerous to mankind. However, little is known regarding indirect toxic effects on the cardiovascular system of respiratory tract exposure to nanometre particles. As a typical nanomaterial, SWCNTs have gained enormous popularity because of their unique properties. However, increasing attention has been paid to the potential pulmonary toxic effects of respiratory tract exposure to SWCNT than to the potential link of this exposure to cardiovascular disease risk. In this study, a rat intratracheal instillation model was used to evaluate the systemic and secondary effects of respiratory tract exposure to SWCNT, specifically changes in lung tissues, the circulatory system and vascular function. We found increased levels of inflammatory factors and interstitial inflammation in the lungs in this rat model. Additionally, up-regulated levels of cytokines and increases in white blood cells, platelets and fibrinogen were detected in the plasma. These changes were followed by increased blood viscosity in the high dose SWCNT exposure group. In addition, damage to the ultrastructure of the vascular intima in the rats were observed. Changes in coagulation and fibrinolysis activating factors were detected in the plasma. Lower expression of t-PA and higher expression of vWF were observed in

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the vascular intima of rats exposed to SWCNT at 10.5 and 17.5 mg/kg b.w for 30 days and 60 days. After exposure to SWCNT for 60 days at 17.5 mg/kg b.w, decreased expression of t-PA gene and increased expression of TM, p38MAPK and PAI-1 genes were observed in the peripheral blood of the rats. Based on these results, we conclude that cardiovascular toxicity caused by respiratory tract exposure to SWCNTs may be induced by indirect effects on vascular homeostasis, which is different from previously reported direct cardiac effects of SWCNT. The present work established a correlation between pulmonary changes and CVD following pulmonary exposure to SWCNT. This study indicates a possible pathophysiological mechanism for CVD caused by pulmonary exposure to SWCNT. More importantly, these results supplement toxicological evaluation data for the risk of CVD caused by respiratory tract exposure to SWCNT.

Keywords: SWCNT; coagulation and fibrinolysis activating factors; vascular ho meostasis; pro-inflammatory factors; lung-mediated cardiotoxicity effects; indirect toxicity; secondary effects

Background

Due to their special structure and physicochemical properties, nanomaterials (NMs) are widely used in, for instance, the chemical, pharmaceutical, and environmental protection fields and electronics.[1-6] An emerging class of environmental pollutants are NMs, and they pose a real threat to human health.[7-10] To date, many countries have implemented a large number of projects and plans with the aim of controlling the risks of nano-technologies. Biological safety research of NMs and the establishment of key evaluation techniques for nano-technologies are necessary to promote the rational use of NMs.[11,12]

Cardiovascular disease (CVD) is a serious danger to mankind. Respiratory tract exposure is one of the main avenues for NMs to enter the human body. Epidemiological studies and experimental data have shown that particulates in polluted air and pulmonary exposure to such particulates can increase the risk and

mortality from CVD[13-18] and that ultrafine particles (nanometre particles) may play a major role in CVD risk.[19-23] However, the systemic effects of pulmonary exposure to nanometre particles and the possible mechanism of cardiovascular disease risk following such exposure are still poorly understood.

Currently, the single-walled carbon nanotube (SWCNT) is one of the most extensively applied NMs in nanotechnology. A large number of studies have focused on the pulmonary toxicity of SWCNT exposure and its mechanism of action.[24-33] In addition, some studies have reported cytotoxic effects and apoptosis in different cell types after exposure to SWCNT.[34-36] Furthermore, several studies regarding the extra-pulmonary effects and systemic effects of pulmonary exposure to carbon nanotubes have been reported.[37-39] Overall, most studies to date have focused on direct damage to tissues, organs or cells after treatment with SWCNT, and little is known about the indirect toxic effects on the cardiovascular system of respiratory tract exposure to SWCNT.

Pro-inflammatory cytokines, such as interleukins and tumour necrosis factor-α (TNF-α), are well-known risk factors for the development of atherosclerosis. Vascular endothelial cells (VECs) are major targets of cytokine signals. Under normal conditions, vascular endothelia is in an antithrombotic and anti-inflammatory state[40], which is key to vascular homeostasis. Endothelial damage is considered the first step in atherogenesis.[41,42] Our preliminary study showed that respiratory tract exposure to NMs cause significant up-regulation of the expressions of inflammatory cytokines in bronchoalveolar lavage fluid.[43]

In this study, a rat intratracheal instillation model was used to evaluate the systemic and secondary effects of SWCNT respiratory tract exposure. In vivo animal experiments were used to examine changes in lung tissues, the circulatory system and vascular function to systematically assess the correlation between respiratory tract immunity injury and the risk of thrombosis. This study provides toxicological evaluation data for the risk of CVD after respiratory tract exposure to SWCNTs.

Methods

Particle Preparation

SWCNTs were purchased from Sigma-Aldrich (St Louis. MO). The tubes were 0.8–1.2 nm in diameter and 0.5-2.0 microns in length, as determined by a transmission electron microscope (TEM) (JEM-2010FEF; JEOL, Tokyo, Japan) (Fig.1A), and the Raman spectra of the functional groups (RM200; Renishaw, Wotton-under-Edge, UK) are shown in Fi.1B. The chemical components were 93.64% C, 1.64% O, 1.60% Ca, 0.98% Fe, 0.62% Co, 0.50% Cr, 0.41% Si, 0.37% S, and 0.25% Cl.
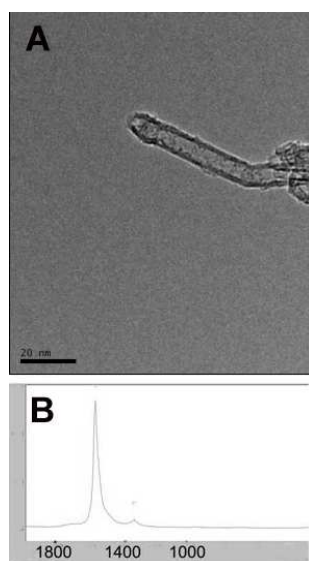

Fig.1. Particle characterisation. A: TEM of SWCNTs. B: Raman spectrum of SWCNTs.

Carbon nanoparticle suspensions were prepared by vortexing carbon nanoparticles in normal saline three times for 5 s and then sonicating them for 6 hours in an ultrasonic bath (KQ2200DE, Shumei, Jiangsu, China) with a water temperature below 40 ☐. Prior to use in the animal experiments, the nanoparticle suspensions were sonicated again for 30 minutes.

Animals and intratracheal instillation treatment

The study protocol was approved by the Chinese Association for Laboratory

Animal Science.

Forty-eight healthy Wistar rats (body weight, 215±8.63 g) were divided randomly into four groups: 1) normal saline control group; 2) 3.5 mg/kg body weight (BW) SWCNT group; 3) 10.5 mg/kg BW SWCNT group; and 4) 17.5 mg/kg BW SWCNT group. Rats were anaesthetised with ether and exposed to the SWCNT suspensions through intratracheal instillation every other day for sixty days. The experiment contained two batches: 6 rats from each group (24 rats total) were randomly selected for sample collection on the thirtieth day of the experiment; the remaining rats continued to be exposed to the SWCNT suspensions every other day until the sixtieth day of the experiment.

Cytokine assays

The levels of interleukin-1α (IL-1α), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) in lung and blood tissues were detected using enzyme linked immunosorbent assay (ELISA) kits (R &D Systems, Minneapolis, MN, USA), and the results were read using an ELISA Reader (Thermo MK3, USA).

Histopathological evaluation

After the rats were euthanised, the left base of the lung and the aorta pectoralis blood vessel were embedded in paraffin and thin-sectioned coronally. The sections were then stained with hematoxylin-eosin for examination by light microscopy.

Detection of hemorheology index and blood constituents

Blood viscosity shear rate, high reduced viscosity, erythrocyte rigidity index, the levels of fibrinogen (FIB) and haematocrit (HCT), and blood platelet (PLT) and white blood cell (WBC) counts were detected using a hemorheology analyser and automatic blood chemistry analyser.

Evaluation of vascular intima ultrastructure by TEM

To evaluate whether damage occurred to the vascular intima of the rats after

exposure to SWCNT through intratracheal instillation, the thoracic aortas of the rats were observed by TEM. Briefly, after exposure to SWCNT for 60 days, the thoracic aortas of the rats were isolated and fixed overnight at 4 °C in 2.5 % glutaraldehyde. Then, the samples were rinsed in 0.1 M phosphate buffer (pH 7.0) three times. Next, the samples were fixed in 1 % osmium tetroxide solution for 2-3 hours and rinsed with phosphate buffer. Then, the samples were dehydrated in gradient ethanol solutions and embedded in Epon-Araldite. Thin sections were counterstained with uranyl acetate and lead citrate for observation. Each experiment was repeated at least three times.

Changes in levels of coagulation and fibrinolysis activating factors

Several molecular markers related to coagulation and the fibrinolysis system in plasma were analysed using ELISA kits (R&D Systems, Minneapolis, MN, USA). The markers included tissue plasminogen activator (t-PA), plasminogen activator inhibitor 1 (PAI-I), D-dimer, antithrombin-III (AT-III), endothelin-1 (ET -1), nitrogen oxide (NO) and von Willebrand factor (vWF). The results were r ead using an ELISA Reader.

Immunohistochemical analyses

To evaluate the expression levels of several important factors in the vascular endothelium, immunohistochemical staining of t-PA, vWF and AT-III antigen in rat vascular intima were performed by the streptavidin-biotin complex (SABC) method. In brief, samples were fixed in 4 % paraformaldehyde for 24 h. The n, the samples were dewaxed, hydrated and PBS-washed. Then, antigen repairin g, blocking, antibody incubation, instillment with the reagent SABC and DAB colouring were performed. Finally, the samples were dehydrated, made transpar ent and mounted. The samples were examined under a fluorescence microscope (OLYMPUS CX41). The data were analysed using BJ43-PAS8000 software. A ll reagents and antibodies were purchased from Abcam.

Isolation of RNA and Real-time polymerase chain reaction (PCR)

Total RNA was extracted from arterious peripheral blood using TRI pure L S Reagent (BioTeke Co.Ltd, Beijing, China). Total RNA amounts were measure d spectrophotometrically. RNA was reverse-transcribed using the Prime Script R T reagent kit (Takara Biotech., Co., Ltd, Dalian, China). RT-PCR was performe d using the Applied Biosystems 7300 System (Life Technologies Co.Ltd, USA) and the SYBR Premix Ex TaqTM II (Tli RNaseH Plus) Kit (Takara Biotech., Co., Ltd, Dalian, China). The reaction mixtures were 20 μl in volume and co ntained 0.4 μM primers. The primers for rat t-PA, PAI-1, thrombomodulin (T M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and p38MAPK were s ynthesised, and the oligonucleotide primer sequences are shown in Table 1. Th e PCR conditions were as follows: incubation at 95 □ for 30 s followed by 4 0 cycles of 95 □ for 5 s and 60 □ for 31 s. mRNA levels were normalised t o GAPDH as a control

Table1. The primers and amplification conditions for RT-PCR

Gene name	Primer Sequences (5'-3')	length of the specific amplification products(bp)	Annealing temperatures(□)
t-PA	F:TCTTCTGTGGAAGAGGAAGAGG R: CTGAACTGGATCCAAGACAACA	95	60
PAI-l	F:TCTCCGCCATCACCAACATT R:GAGAGAACTTAGGCAGGATGAGG	99	60
TM	F:GAAACCTTCCTGGCTCCTATG R:GGGGTCACAGTCCTTGCTAAT	80	60
p38MAPK	F:AGACCGTTTCAGTCCATCATTC R:ACACATCCAACAGACCAATCAC	100	60
GAPDH	F:GGCACAGTCAAGGCTGAGAATG R:ATGGTGGTGAAGACGCCAGTA	143	58

F:Forward; R: Reverse.

Statistical analysis

Data are expressed as means ± standard deviation. All experiments were repeated at least three times. The statistical analyses were performed using SPSS software. The results were examined by ANOVA, followed by the least post squares (post hoc test)

(equal variances) or Dunnet's T3 post hoc test (unequal variances). Values of $p < 0.05$ were considered to be statistically significant.

Results

Changes in cytokine levels in lung and blood tissues

Inflammatory cytokines IL-1α, IL-6 and TNF-α play important roles in regulating immunity. After exposure to varying doses of SWCNT for 30 and 60 days, the levels of IL-1α, IL-6 and TNF-α in lungs and blood of rats tended to increase as compared to those in the control groups at those two time points (Fig.2). The level of IL-1α increased significantly in the lungs of rats after exposure to SWCNT at 10.5 mg/kg b.w for 60 days. At 30 days and 60 days after exposure to the SWCNT dose of 17.5 mg/kg b.w, the level of IL-1α in the lungs of rats increased significantly ($p < 0.05$) (Fig.2A). At 60 days after exposure to the SWCNT dose of 17.5 mg/kg b.w, the level of IL-6 increased significantly in the lungs of rats ($p < 0.05$) (Fig.2B). Compared with the control group, the level of TNF-α increased significantly in the lungs of rats after exposure to SWCNT at 3.5, 10.5 and 17.5 mg/kg b.w for 30 days, and the level of TNF-α showed similar increasing trends after exposure to SWCNT at 10.5 and 17.5 mg/kg b.w for 60 days ($p < 0.05$) (Fig.2C). Figure 3 shows the levels of cytokines in the blood of rats after SWCNT exposure. After exposure to SWCNT at 17.5 mg/kg b.w for 30 and 60 days, the levels of IL-1α and IL-6 in the blood were significantly higher than those in the control group at those two time points ($p < 0.05$) (Fig.3A-B). Additionally, the levels of TNF-α were significantly higher in the blood of rats after exposure to SWCNT at 10.5 and 17.5 mg/kg b.w for 60 days ($p < 0.05$) (Fig.3C).
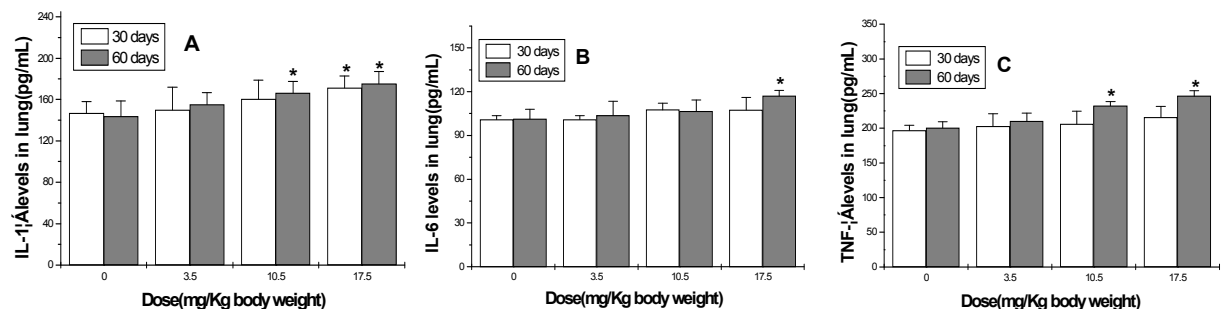
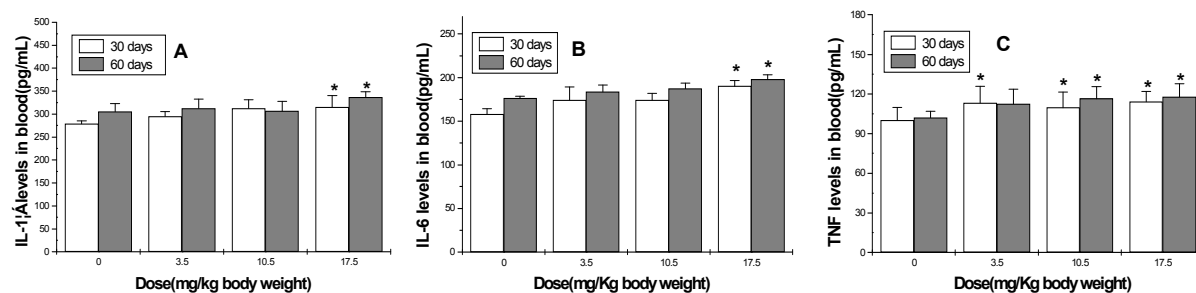Fig.2. Several inflammatory markers in lung induced by SWCNTs. *p<0.05 compared with control.

Fig.3. Several inflammatory markers in plasma induced by SWCNTs. *p<0.05 compared with control.

Histopathological evaluation

Fig.4 shows the results of the pathological evaluation of the lung tissues and endangium of the rats. After exposure to SWCNT through intratracheal instillation for 30(Fig.4.1a-1d) and 60(Fig.4.2a-2d) days, the lungs of the rats in the exposure groups showed pathological changes. The lung tissues showed brownish black particle deposition and mild to moderate alveolar and local interstitial inflammation. Moreover, with increasing doses of SWCNT (from 3.5 to 10.5 to 17.5 mg/kg b.w) and the extension of the exposure time, the degree of pathological injury increased in a dose/time-dependent manner. There were no significant pathological change in endangium of rats after exposed to SWCNT at 3.5 and 10.5 mg/kg b.w for 60 days. But, the endangium of rats showed mild infiltration of inflammatory cells after exposed to SWCNT at 17.5 mg/kg b.w for 60 days(Fig.4.3a-3d).
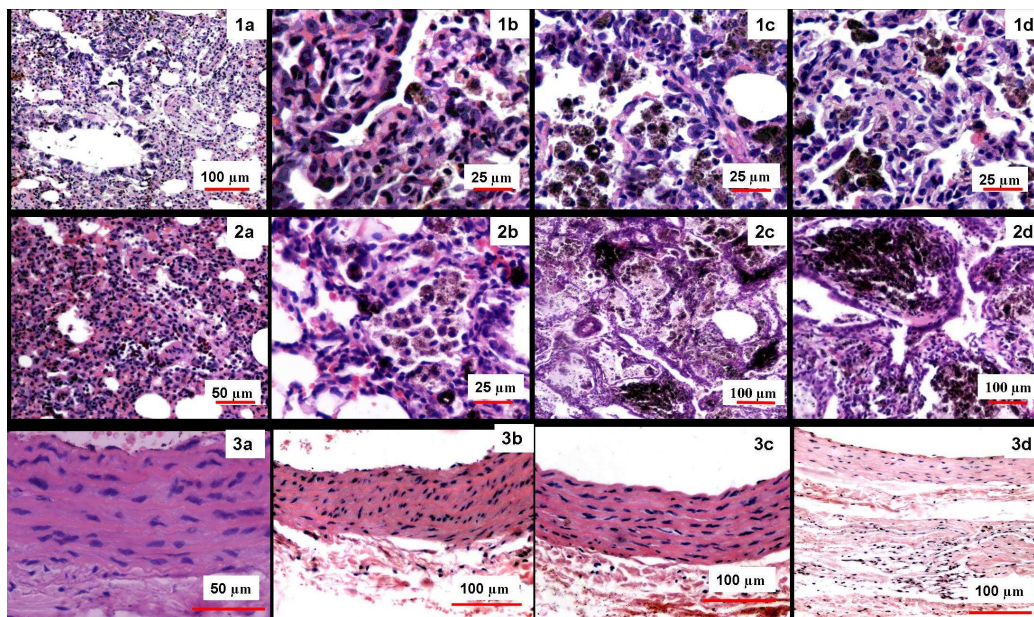
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Fig.4. Pathological changes in rats exposed to SWCNTs. 1a-1d and 2a-2d: Lung tissue exposure for 30 days and 60 days, respectively. 3a-3d: Endangium of aorta pectoralis exposure for 60 days. a: control; b, c and d: 3.5, 10.5 and 17.5 mg/kg b.w, respectively.

Detection of hemorheology index and blood constituents

The related hemorheology indexes were detected in the rats after exposure to SWCNT(Fig.5). Compared with the control group, blood viscosity shear rate, high reduced viscosity and erythrocyte rigidity index in the high SWCNT dose group (17.5 mg/kg b.w) increased significantly after exposure to SWCNT for 60 days ($P<0.05$) (Fig.5A-C). The raised erythrocyte rigidity index indicated less erythrocyte degeneration, which was a major factor in the increase in blood viscosity under the high shear rate. The plasma fibrinogen level significantly increased as compared to that of the control group after 30 days of exposure to SWCNT at 17.5 mg/kg b.w. Exposure to SWCNT at all doses for 60 days resulted in significant increases in plasma fibrinogen level ($P<0.05$) (Fig.5D).
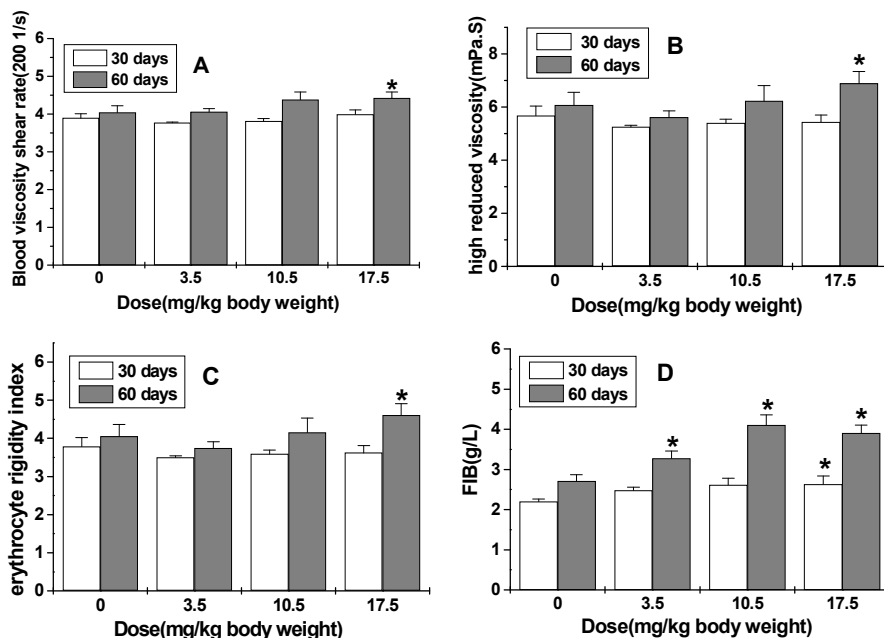
Fig.5 Seveal indices of hematology in the plasma of rats. *$p<0.05$ compared with control.

Fig.6 shows changes in HCT, PLT and WBC levels in rat plasma after exp osure to SWCNT. The levels of HCT in rat plasma tended to increase after ex posure to SWCNT for 30 and 60 days. The HCT level in the 3.5 mg/kg b.w SWCNT group after exposure for 60 days and that in the 17.5 mg/kg b.w SW CNT group after exposure for 30 days were significantly higher than that in th e control group ($p<0.05$) (Fig.6A). After exposure for 30 days, the PLT levels in the 3.5 and 10.5 mg/kg b.w SWCNT groups increased significantly compar ed to that of the control group ($p<0.05$) (Fig.6B). Compared with the control group, the WBC levels increased significantly in all experimental groups after exposure for 60 days ($p<0.05$). After exposure for 30 days, a significant incre ase in WBC level was only observed in the high dose group (17.5 mg/kg b.w) ($p<0.05$) (Fig.6C).
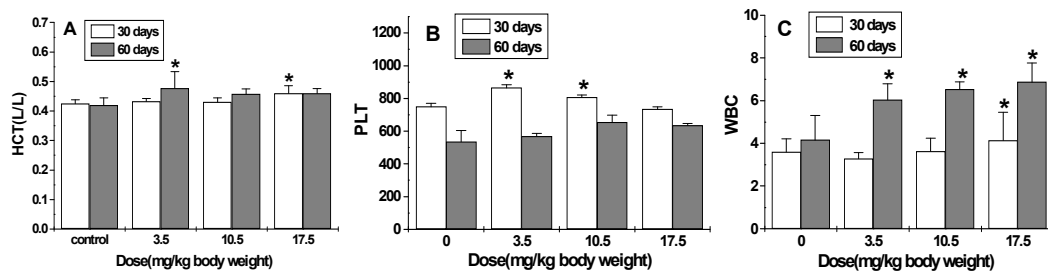
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Fig.6. Levels of HCT, PLT and WBC in the plasma of rats. *p<0.05 compared with control.

Changes in levels of coagulation and fibrinolysis activating factors in rat plasma

Under physiologic conditions, VECs function mainly to prevent thrombosis t o ensure smooth blood flow and the maintenance of the circulation pipeline. When the vessel wall experiences damage or dysfunction, the levels of coagula tion and fibrinolysis activating factors that are secreted, synthesised and release d by VECs may also change. In this experiment, a variety of molecular marke rs of the prethrombotic state were tested in rat plasma, and the results are sho wn in Fig.7. In the 10.5 and 17.5 mg/kg b.w SWCNT groups at 30 and 60 d ays, t-PA levels were significantly lower than that in the control group (P<0.05) (Fig.7A). The PAI-1 level was significantly increased in the 10.5 mg/kg b.w SWCNT group after 30 days and in the 17.5 mg/kg b.w groups after 30 and 60 days as compared to the control group (P<0.05) (Fig.7B). After 60 days of exposure, the vWF levels in the 10.5 and 17.5 mg/kg b.w SWCNT groups w ere significantly higher than that in the control group (P<0.05) (Fig.7C), and th e ET-1 levels in the 17.5 mg/kg b.w SWCNT group were significantly higher t han that of the control group (P<0.05) (Fig.7D). The D-dimer levels were incr eased significantly in the 10.5 mg/kg b.w SWCNT group after 60 days and in the 17.5 mg/kg b.w SWCNT group after 30 and 60 days as compared with t hose in control group at the same time points (P<0.05) (Fig.7E). AT-III levels in the plasma were decreased after exposure to SWCNT. There were significant differences between the exposure groups at all doses of SWCNT and the cont

rol group, except for the 17.5 mg/kg b.w SWCNT group after 60 days (*P*<0.0 5) (Fig.7F). NO levels were decreased significantly in all SWCNT exposure gr oups as compared to that of the control group at 60 days (*P*<0.05) (Fig.7G).
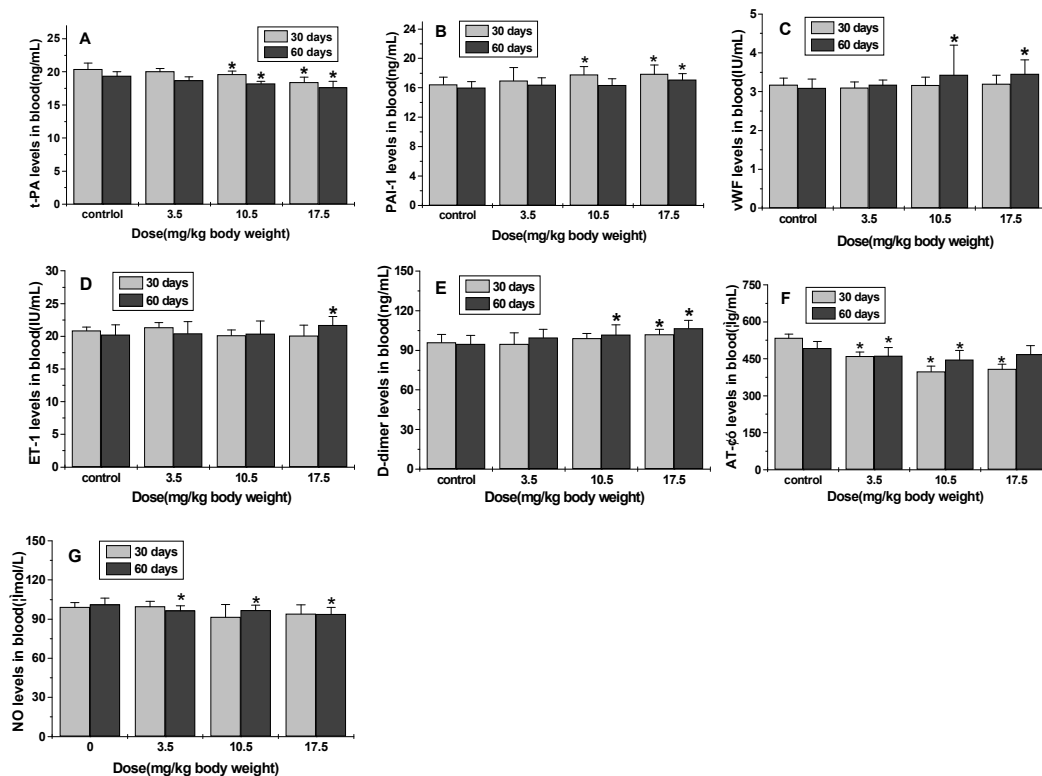

Fig.7. Levels of coagulation and fibrinolysis activation factors in plasma. *p*<0.05 compared with control.

Immunohistochemical analyses

Fig.8 shows the expressions of t-PA, vWF and AT-III in the vascular intima of rats visualised under a microscope. The positive areas were analysed, and t he results are shown in Fig.9. The expressions of t-PA in the vascular intima of rats in all SWCNT exposure groups were significantly lower than that in th e control group at 30 and 60 days (*P*<0.05) (Fig.9A). The level of AT-III incr eased significantly after exposure to SWCNT at 3.5 mg/kg b.w for both 30 a nd 60 days and after exposure to SWCNT at 10.5 mg/kg b.w for 30 days. Ho wever, at the higher SWCNT dose of 17.5 mg/kg b.w, the levels of AT-III dec reased significantly compared with those of the control group at both 30 and 6

0 days (*P*<0.05) (Fig.9B). Thus, lower doses of SWCNT may stimulate the vascular intima to synthesis more AT-III than higher doses. However, the increased dose of SWCNT resulted in damage to the vascular intima, which led to a decrease in the expression of AT-III. The levels of vWF were significantly higher in the plasma of rats exposed to SWCNT at 10.5 and 17.5 mg/kg b.w for 30 and 60 days as compared to those in the control group at 30 and 60 days (*P*<0.05) (Fig.9C).
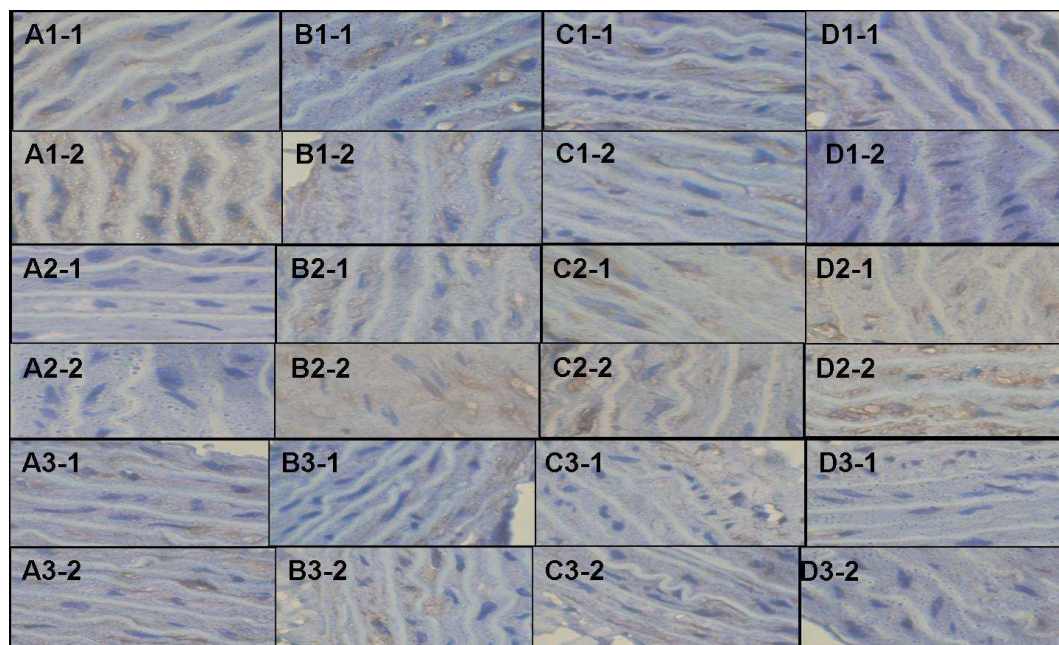

Fig.8. Immunohistochemical images. A1-D1:t-PA; A2-D2:vWF; A3-D3:AT-□.
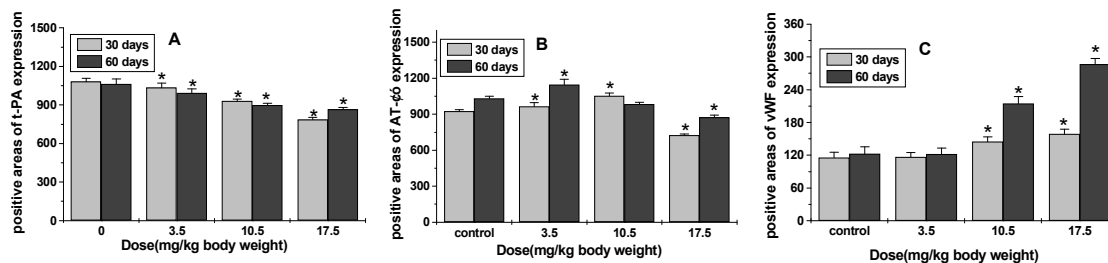-1：exposure for 30 days; -2: exposure for 60 days.

Fig.9. Results of immunohistochemical analysis. **p<0.05* compared with control.

Ultrastructural changes to the vascular intima of rats

Fig.10 shows the TEM results. After respiratory exposure to SWCNT for 60 days, nuclear malformation occurred in the vascular endothelial layer of rats exposed to SWCNT at 3.5 mg/kg b.w. The vascular endothelial layer of rats in the 10.5 mg/kg b.w SWCNT group showed mitochondrial swelling and cytoplasmic shrinkage. Additionally, mitochondrial vacuolisation was observed in the 17.5 mg/kg b.w SWCNT group.
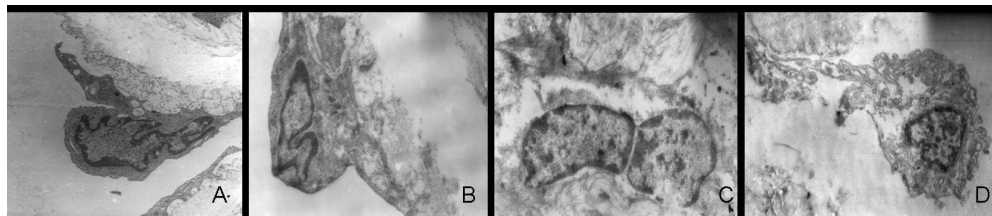
Fig.10. TEM of aortic vascular intima of rats exposed to SWCNT for 60 days. A (8000×): control; B (10000×): 3.5 mg/kg b.w; C (8000×): 10.5 mg/kg b.w; D (4000×): 17.5 mg/kg b.w.

Gene expression in peripheral blood of rats

Peripheral blood cells play an important role in the maintenance of the internal environment. In this experiment, several related genes in the peripheral blood were detected to determine possible markers of SWCNT toxicity. As shown in Fig.11, t-PA gene expression was decreased and TM and p38MAPK gene expressions were increased significantly after exposure to SWCNT for 60 days at 17.5 mg/kg b.w as compared to the expression of those genes in the control group ($P<0.05$) (Fig.11A-C). PAI-1 gene expression increased after exposure to SWCNT as compared to that in the control group; however, this difference was not significant (Fig.11D).

Fig.11. Gene expression in peripheral blood of rats exposed to SWCNT for 60 days. *$p<0.05$ compared with control.
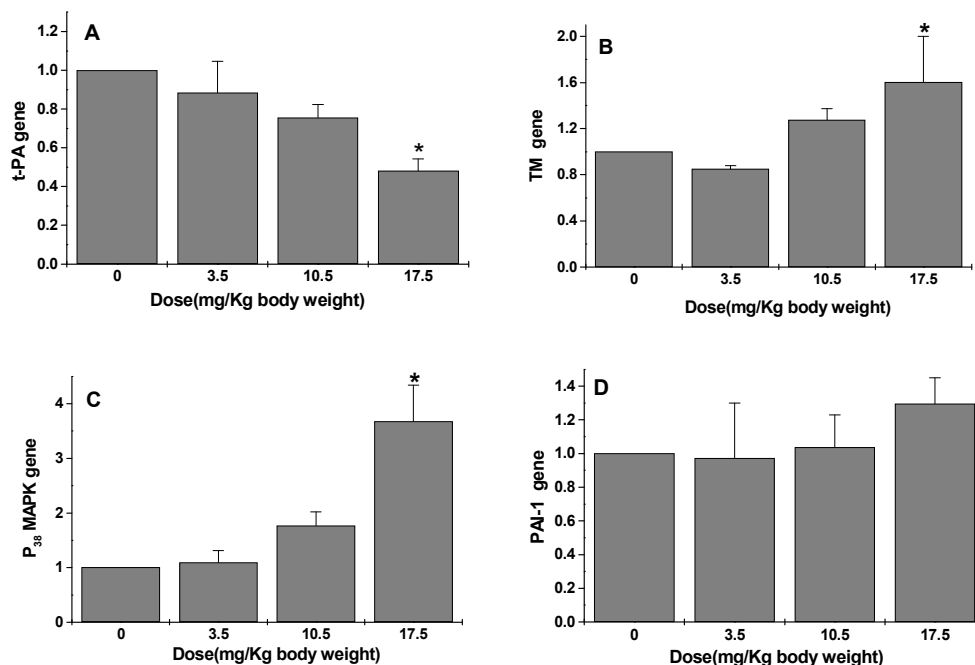
Discussion

In this study, an animal model of rat intratracheal instillation was used to si
mulate respiratory tract exposure to SWCNT. After exposure to SWCNT, the e
xpression levels of inflammatory factors in the lung tissues of rats increased si
gnificantly as compared to those in the lung tissues of the control group, indic
ating an inflammatory immune response in the lung tissues of the rats exposed
to SWCNTs. These results were consistent with those of previous studies of t
he pulmonary toxicity of NMs.[33,44] We also observed varying degrees of interst
itial inflammation and focal brown agglomerates in the lung tissues of the rats
exposed to SWCNTs. Macrophages that had engulfed SWCNTs were shown to
have gathered in the endotracheal tissues of the rats exposed to SWCNTs. Be
cause SWCNTs were not identified and effectively removed by the alveolar ma
crophages, they diffused from the agglomerates into alveolar cavities where the
y could persist. This could help the SWCNTs enter the systemic circulation thr

ough the local pulmonary blood barrier,[45,46] however, Matthews et al.[47] reported that no more than 0.05% of a dose of SWCNT instilled over 90 min translocated from the airways across an intact pulmonary barrier into the systemic circulation. Thus, the persistence of the local inflammatory injury caused by SWCNTs is more important than the movement of SWCNTs into the systemic circulation, as it greatly increases the release of inflammatory mediators produced locally in the lungs into the systemic circulation, resulting in an amplification of the inflammatory reaction throughout the body.

Van et al. found that pulmonary inflammation caused by contact with particulate matter air pollutants can up-regulate the levels of cytokines in the blood circulation.[48-51] In this study, after respiratory exposure to SWCNT for 30 and 60 days, rats presented with systematic inflammation of varying degrees, which manifested as elevated inflammatory factor plasma levels, increased white blood cell and platelet levels in the blood and increased blood viscosity and fibrinogen content in the blood. These results indicate that NMs, herein represented by SWCNTs, have toxicities that are not limited to lung injury in rats exposed to them through the respiratory tract. In the current study, we focused on the changes in systemic inflammation and blood viscosity after lung injury caused by NMs and whether this type of secondary toxicity of NMs affects the vascular system and, thus, increases the risk of cardiovascular disease.

Thus, in the current study, the ultrastructure of the vascular intima of rats was observed. We found abnormal changes in the vascular endothelial layers, such as cell nuclear malformation and cytoplasmic shrinkage after exposure to SWCNT for 60 days. Additionally, pathological examination revealed inflammatory cell infiltration around the vascular intima after high dose exposure to SWCNT for 60 days. All these results suggest that the vascular intima was stimulated by SWCNT resulting in different degrees of damage.

Normal vascular intima has anticoagulant and antifibrinolytic physiological functions through the synthesis, secretion and release of a variety of blood coagulation and fibrinolysis activating factors, which serve to balance and regulate

thrombosis and inflammation. When the vascular intima is stimulated by physical factors, chemical factors and other factors, such as inflammatory factors, it may be damaged or disordered resulting in changes in the levels of coagulation and fibrinolysis activating factors. Coagulation and fibrinolysis activating factors are synthesised and secreted by VECs and include t-PA, PAI-I, AT-Ⅲ, vWF and ET-1. t-PA is a serine protease that converts plasminogen to its active form, the proteinase plasmin. PAI-I is a glycoprotein that is a major physiological inhibitor of plasminogen activators.[52] The regulation of fibrinolysis activity in the plasma mainly depends on the relative proportion of t-PA/PAI-1 secreted by VECs. In this study, both the level of t-PA in the plasma and the expression of t-PA antigen in the vascular intima were significantly lower in the SWCNT exposure groups than those in the control group. Furthermore, the level of PAI-1 in the plasma of the rats of the SWCNT exposure groups was significantly higher than that in the control group. All these results indicate that antithrombotic function of VECs was weakened by SWCNT exposure. The D-dimer plasma level was significantly higher in the high dose SWCNT group than in the control group. D-dimer is a fibrin degradation product and a specific marker of fibrinolysis. Some pathological conditions, such as infection, intravascular activated thrombosis and secondary fibrinolysis hyperfunction, could lead to an increase in D-dimer in the plasma.

AT-Ⅲ is a multifunctional serine protease inhibitor that can form complexes with thrombin to inactivate it. Under physiological conditions, AT-Ⅲ exerts its anticoagulant effect on the surface of VECs by being secreted and bound by VECs continuously; this allows AT-Ⅲ to inactivate coagulation factors that are activated and presented on the surface of the vascular endothelium in a timely manner. When blood vessels are subjected to external stimuli, such as the inflammatory factor effect, AT-Ⅲ synthesis by VECs is reduced. In our experiment, the level of AT-Ⅲ in rat plasma decreased significantly after exposure to SWCNT. However, the expression of AT-Ⅲ antigen in the vascular intima increased with the lower doses of SWCNT and decreased with the highest dose of SWCNT. This may be because alterations in the blood components after low dose exposure to SWCNT stimulated VECs to synthesise

AT-Ⅲ on their surfaces, while high dose exposure to SWCNT generated vascular intima injury, resulting in a reduction of the synthesis of AT-Ⅲ. VWF and ET-1 are molecular markers of VEC injury. The expression of VWF in the vascular intima of rats increased significantly after SWCNT exposure. Moreover, the plasma levels of vWF and ET-1 in the high dosage SWCNT group were elevated significantly. All these results further validated the injury of VECs in rats cause by SWCNT exposure.

Studies of gene expression in the peripheral blood can reflect whether the body is in a state of health or disease, which has important significance for the prediction of cardiovascular effects caused by NMs. In this study, the expression of several related genes in the peripheral blood were examined. Among them, TM is a glycoprotein that presents on the vascular inner surface, and it can activate protein C by combining with thrombin and blocking the blood coagulation system. In the high dose SWCNT exposure group, t-PA gene expression was lowered and TM gene expression was increased significantly as compared to their expression in the control group. Thus, t-PA and TM gene expression could be sensitive biomarkers for respiratory tract exposure to SWCNT. Additionally, *p*38MAPK gene expression was obviously higher in the high dose groups, suggesting that the *p*38MAPK signalling pathways are involved in the mechanism of action of changes in coagulation and fibrinolysis activating factor levels in the body. Further research regarding biological marker genes and related signalling pathways are necessary.

Brook et al.[14,53] had reviewed that the release of pro-inflammatory mediators or vasculoactive molecules from lung based cells was one of pathways linking Particulate Matter and CVD. In the present study, we systematically evaluated the correlation between pulmonary immune injury and damage to the vascular system. The biological pathways that were examined are shown in Fig.12. As a target organ, the lung had experienced inflammatory immune injury after exposure to SWCNT through the respiratory tract. Pro-inflammatory factors were generated in local pulmonary tissue and could be released into the systemic circulation, thus, raising circulating levels of cytokines and influencing platelet production, blood coagulation, and other cardiovascular factors. The amplification

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of this local inflammation leads to effects on the vascular system, including v ascular inflammation, endothelial dysfunction in regards to coagulation or antico agulation and vascular intima injury. These effects on the vascular system even tually disrupts vascular homeostasis, thus, indirectly mediating changes in coagu lation and fibrinolysis in the body. This is an important pathogenic mechanism that is different from the direct cardiac toxicity effects of SWCNT reported b y previous studies. The vascular homeostasis imbalance could cause the increas ed incidence and mortality of cardiovascular.

At present, oxidative stress and mitochondrial damage caused by ROS are considered the main mechanisms of the toxic effects of NM on the cardiovascular system. However, as initiating factors, inflammatory factors participate in vascular injury and regulate changes in both the coagulation and fibrinolysis systems of organisms. The results of our preliminary experiment suggest that there are other related signalling pathways involved in the indirect effects on the cardiovascular system of NM exposure to the lungs. In our subsequent research, we will use an in vitro cell co-culture model to further examine the mechanism of these indirect toxic effects of NMs on the cardiovascular system.

Conclusions

Our research indicated that after respiratory tract exposure to SWCNT, in addition to inflammatory immune injury in the lung, systemic inflammation, vascular endothelial dysfunction and vascular endothelial damage were observed in rats. The rats entered a prethrombotic state, which increased their risk of CVD by increasing their risk of thrombosis and atherosclerosis. SWCNT can induce cardiovascular toxicity through indirect effects on vascular homeostasis. Moreover, the release of pro-inflammatory factors from sites of pulmonary injury plays a key role in these indirect effects of SWCNT and may be a major pathophysiological mechanism of the lung-mediated cardiotoxicity effects of SWCNT.
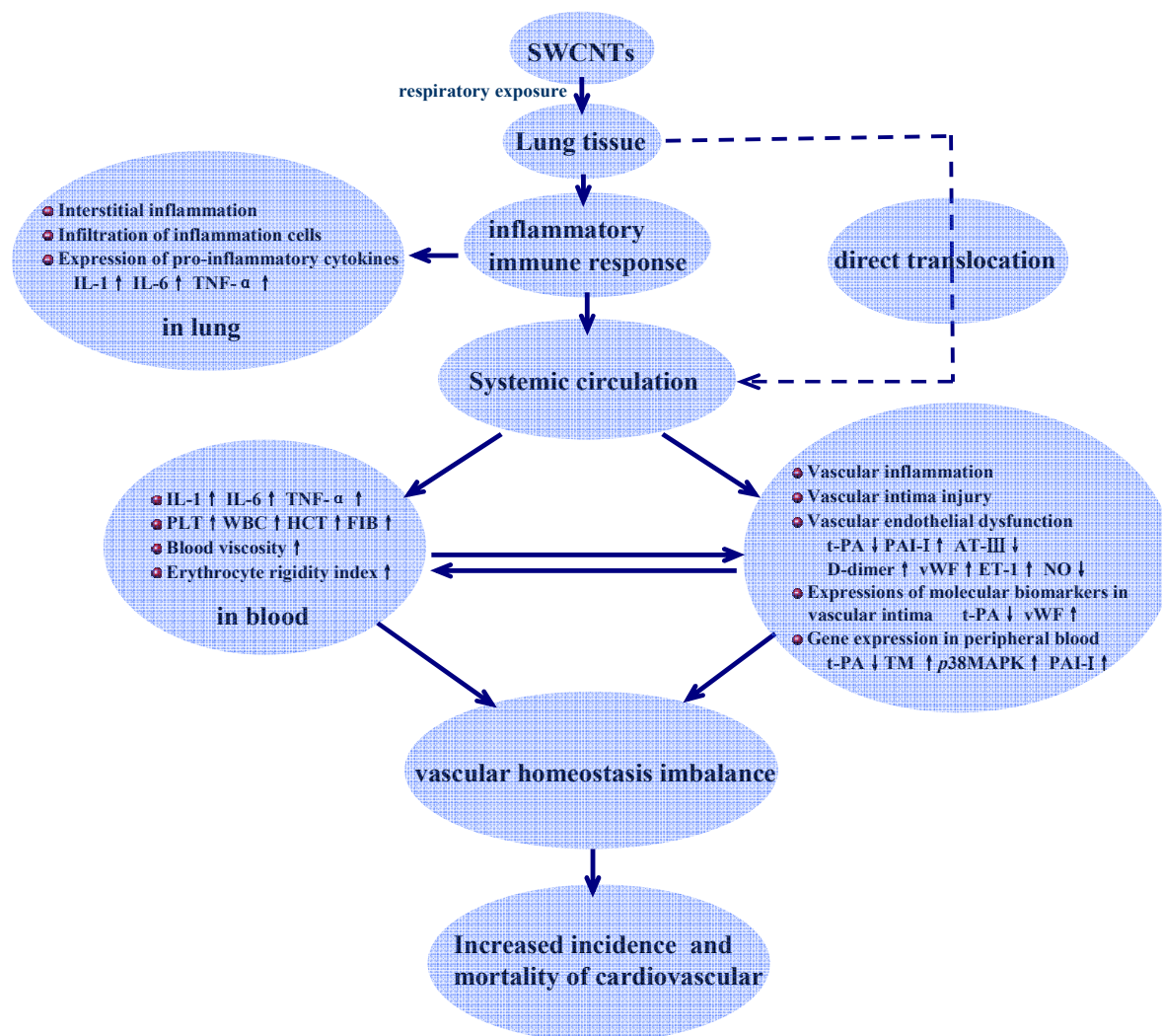
Fig.12. The biological pathways linking SWCNT and CVD.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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