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奈米微粒與健康風險研究-子計畫五：疾病動物模式奈米微粒毒性探討

計畫類別：□ 個別型計畫　　■ 整合型計畫

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執行單位：國立台灣大學職業醫學與工業衛生研究所

中　華　民　國　　年　　月　　日

奈米微粒與健康風險研究-子計畫五：疾病動物模式奈米微粒毒性探討

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摘要

流行病學研究指出，空氣污染氣懸微粒的增加，與呼吸系統疾病和心血管疾病死亡率增加有關，這些死亡大都發生於已經有心肺疾病的個人，然而確實致病機轉仍不清楚。近年研究發現小粒徑微粒可能扮演更重要的角色，特別是奈米微粒在同一重量濃度下，顆粒數變大，表面積也加大，可能增加毒性，目前已有一些研究針對奈米微粒與細胞毒性的關係進行探討，主要著重在奈米微粒造成的發炎反應，但是對於奈米微粒引起的呼吸道變化及心血管系統效應之間的關係並不清楚，特別在易感性族群的健康效應研究更少。本研究目的為：(1)探討肺高血壓疾病動物暴露奈米聚苯乙烯後，肺部發炎與傷害反應，及氧化壓力的產生，同時也探討這些指標與表面積之相關。(2)以糖尿病大鼠模式進行奈米微粒心血管毒性試驗，以氣管灌注方式暴露奈米碳黑，觀察其肺部發炎及周邊血液發炎反應與內皮細胞功能標記之變化。(3)探討自發性高血壓大鼠與健康 SD 大鼠在暴露奈米微粒後，肺部發炎與氧化傷害之比較。(4)探討非細胞系統中奈米微粒粒徑與氧化壓力之相關。(5)探討肺部上皮內襯液體（ELF）對於奈米微粒引起細胞氧化傷害的影響，評估奈米微粒與細胞反應後產生之氧化壓力及評估細胞 DNA 單股斷裂情形。

研究結果顯示，肺高血壓大鼠暴露微粒後，暴露小粒徑微粒的動物有較高的肺部發炎及傷害反應，同時這些反應與總表面積有關，另外氧化壓力指標也有類似趨勢。我們也發現糖尿病大鼠及健康大鼠，暴露於奈米碳黑都導致顯著的肺部發炎及傷害反應，但是奈米碳黑只在糖尿病大鼠造成顯著的周邊發炎反應增加，血管內皮素升高及血液一氧化氮降低，奈米微粒暴露可能與糖尿病有共同的的病理生理作用途徑，造成心血管疾病增加的風險。進一步研究比較自發性高血壓大鼠與健康 SD 大鼠的肺部發炎與氧化壓力，自發性高血壓大鼠因抗氧化壓力能力較差，所以不管在肺部發炎反應或氧化壓力指標都比較健康 SD 大鼠明顯。因為奈米微粒之毒性與氧化壓力有關，所以進一步在非細胞系統中，測試奈米微粒氧化壓力的產生，發現奈米碳黑產生的氧化壓力隨暴露濃度及暴露時間增加，同時與總表面積有關。而碳黑微粒在添加 ELF 的環境下能顯著降低氧化壓力的產生，同時減少 DNA 單股斷裂的產生。

我們的研究顯示，相同重量下，同材質的奈米微粒毒性較粗微粒高，可能與奈米微粒的數目及總表面積皆較粗微粒高，引起較大的氧化壓力有關；奈米微粒除了造成肺部疾病，可能也與心血管疾病有關，特別是易感性的糖尿病者，奈米微粒的毒理效應更大，研究也發現氧化壓力在微粒導致的肺部及心血管疾病，扮演重要角色。另外，我們發現非細胞系統也可進一步發展成篩檢的工具。研究顯示奈米粒徑微粒可在大氣微粒扮演重要角色，同時在奈米材料毒性研究上，也提供有用的資訊，有關奈米微粒毒理機轉，有待進一步研究。

本研究報告將依照年度分為三部分，第一部份為聚苯乙烯對肺高血壓大鼠之肺部發炎反應研究。第二部份為糖尿病大鼠暴露奈米微粒之心血管疾病研究，以及肺部上皮內襯液體（ELF）對於奈米微粒引起細胞氧化傷害及細胞 DNA 單股斷裂的影響。第三部份為奈米微粒於非細胞系統之氧化壓力反應，還有自發性高血壓大鼠與健康 SD 大鼠的肺部發炎與氧化壓力之比較。

關鍵詞：奈米微粒，心肺毒性，氧化壓力，肺高血壓大鼠，糖尿病大鼠，自發性高血壓大鼠，內皮細胞功能，碳黑，聚苯乙烯

Abstract

Epidemiologic studies have shown consistent associations between the exposure to particulate air pollution and acute increase in morbidity and mortality, especially for susceptible subjects with pre-existing respiratory and cardiovascular disease. However, the exact mechanism remains unclear. Recent studies have shown that ultrafine particles may have a greater inflammatory effect than larger particles at the same mass concentration because of larger surface area and oxidative stress. However, the role of ultrafine particles on cardiopulmonary events is not clear. The goal of this study was: (1) to investigate the lung inflammation and oxidative

stress in pulmonary hypertensive rats exposed to polystyrene particles. (2) to evaluate the effect of ultrafine carbon black on lung inflammation, systemic inflammation and endothelial dysfunction in STZ-diabetic rats. (3) to compare the lung inflammation and oxidative stress between spontaneously hypertensive rats and healthy SD rats. (4) to investigate the oxidative stress formation in a cell free system exposed to carbon black nanoparticles. (5) to investigate the effect of epithelium lining fluids (ELF) on ultrafine carbon black-induced ROS generation and DNA single strand breaks. Our results revealed that polystyrene nanoparticles can induce greater lung inflammation and injury and oxidative stress as compared to larger particles. The outcome parameters and total surface area were also highly correlated. Carbon black nanoparticles induced lung inflammation in both DM and non-DM rats, while abnormal endothelial function was only observed in DM rats. We further compared the lung inflammation and oxidative stress between SHR and SD, and found that those parameters were greater in SHR. In cell free system, the amounts of ROS increased with exposure concentration and exposure time. ELF significantly decreased ROS and DNA SSB. Our results indicate that nanoparticles can induce oxidative stress, which may be related with subsequent cardiopulmonary changes. The results also found that diseased subjects were more susceptible to nanoparticles. It is interesting to know that nanoparticles and diabetes may share the common pathway leading to cardiovascular events. We also find that cell free system may be used to screen nanoparticles.

Keywords: nanoparticles, diabetic rats, epithelium lining fluid, reactive oxygen species, cardiopulmonary disease, endothelial function, spontaneously hypertensive rats, pulmonary hypertension, carbon black, polystyrene

奈米微粒與健康風險研究-子計畫五：疾病動物模式奈米微粒毒性探討 (I)

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中文摘要

目的

早期對於奈米微粒的毒性研究著重在金屬作業場所，通常探討工人暴露於會引起金屬燻煙熱的奈米燻煙微粒所造成的健康效應。最近的流行病學研究指出，空氣污染中的大氣懸浮微粒暴露與心肺疾病死亡率增加有關，相關研究指出，大氣奈米微粒可能是呼吸與心血死亡率增加的重要原因之一，然而機制並不清楚。奈米技術已成為高科技產業的主軸，製程中產生的奈米微粒對健康產生的影響並不清楚，因此有必要針對奈米微粒毒性進行系統性的研究。本研究以低毒性的聚苯乙烯奈米微粒進行細胞及動物實驗，探討不同粒徑、數目濃度之奈米微粒造成的毒理效應。

方法

奈米聚苯乙烯微粒粒徑範圍包括 64、109、535-nm，細胞實驗方面，以人類第二型肺泡上皮細胞(A549)進行粒徑為 64、109、202、535-nm 之奈米微粒 100μg/ml 及 1mg/ml 各 4 小時之暴露，以 ELISA 測定發炎前趨物細胞激素 IL-8 及 IL-6 之表現。動物實驗則以肺高血壓疾病動物模式進行，以氣管灌注 0.5mlPBS 生理緩衝液為控制組，0.5ml 100μg/ml 及 50μg/ml 的 64、109、202、535-nm 奈米微粒為暴露組，暴露後 24 小時將大鼠犧牲，收集肺灌洗液、組織及周邊血液，進行肺部發炎指標及氧化壓力指標測量。

結果

細胞實驗方面，人類第二型肺泡上皮細胞 A549，暴露於 100μg/ml 奈米微粒組有較高之發炎前趨細胞激素 IL-8 表現。動物實驗方面，與對照組比較，暴露於 64-nm 奈米微粒之肺高血壓大鼠，肺泡灌洗液之總細胞數及嗜中性球比例、及總蛋白質皆顯著高於同質量濃度下較大粒徑的暴露組，同時肺部灌洗液產生的細胞激素 IL-6 亦高於對照組，代表暴露於奈米微粒的疾病動物肺部有顯著的發炎反應。另外，暴露於 64-nm 奈米微粒之大鼠，其肺部組織 GSH 則較對照組有明顯降低的趨勢，與奈米微粒的表面積有劑量反應關係；同時周邊血液的氧化壓力產物 8-OHdG 亦隨著奈米微粒反應表面積的增加而上升，顯示暴露於奈米微粒後會產生較高之氧化壓力。

結論

結果顯示，暴露於低毒性的奈米微粒會造成肺高血壓大鼠肺部損傷，並且引發氧化壓力，造成顯著發炎反應，本研究建議針對其他材質的奈米微粒毒性及毒理機制進一步研究。

關鍵字：奈米微粒，人類肺泡上皮細胞，肺高血壓大鼠，發炎指標

Abstract

Objective

The effects of air pollution particles on cardiopulmonary mortality and morbidity have been well documented. However the role of ultrafine particles on the mechanism of lung injury is unclear. The aim of the present study is to investigate inflammatory responses and oxidative stress of ultrafine polystyrene particles on disease animal model and alveolar cell lines.

Method

MCT-induced pulmonary hypertension rats were exposed to 100μg/ml and 1 mg/ml of 64-nm polystyrene nanometer particles by intratracheal instillation and sacrificed after 24h. Peripheral blood and brochoalveolar lavage fluid were collected for analysis of inflammation and oxidative stress markers. Further, A549 epithelial cells were exposed of 100μg/ml and 1 mg/ml of 64, 109 and 202-nm ultrafine polystyrene particles for 4 hours, respectively. Cytokine IL-6 and IL-8 were measured using ELISA.

Results

In the pulmonary hypertensive rats model, we demonstrated that there was a significant increase in total cells and percentage of neutrophils ($p<0.05$) in the rat lung after intratracheal instillation of

100μg/ml of 64-nm polystyrene particles compared with control rats. The total protein in bronchoalveolar lavage fluid showed a significant increase after treatment of 64-nm particles than controls ($p<0.05$). The depletion of glutathione (GSH) in the lung tissue was also observed in rats after the instillation of 64-nm ultrafine particles as compared with the control. We also demonstrated that higher cytokine IL-6 ($p<0.05$) and TNF-α in rats after instillation of 64-nm ultrafine particles. We observed that 100μg/ml of ultrafine polystyrene particles induced higher levels of cytokine IL-8. However, IL-6 level were not increase after the particle treatment.

Conclusion

These findings suggest that ultrafine particles composed of low toxicity materials such as polystyrene may cause lung inflammation and oxidative stress in disease animal model, even at lower concentration.

Key words: nanometer particles, A549, pulmonary hypertensive rats, inflammation

Introduction

Epidemiologic studies have shown consistent associations between the exposure to particulate air pollution and acute increase in morbidity and mortality, especially for susceptible subjects with pre-existing respiratory and cardiovascular disease (Pope et al., 2002; Pope and Dockery 1999; Samet et al., 2000). These epidemiologic studies also document that particles with a diameter below 2.5μm ($PM_{2.5}$) have greater adverse health outcomes as compared to particles with a diameter below 10μm (PM_{10}). Recent studies further indicate that the ultrafine particles (< 100 nm), which by count constitute the majority of urban particulate air pollution, may have an increased toxicity relative to larger particles under the same mass concentration (Ferin et al., 1992; Oberdorster et al., 1995; 2001).

One explanation of ultrafine particle toxicity is the production of oxidative stress. Reactive oxygen species (ROS) may be generated through the interaction between surface area of ultrafine particles and target cells, such as macrophages and epithelial cells. Then, ROS may elicit transcription of proinflammatory cytokines and result in a cascade of inflammation events (Stone et al., 1998). Many studies suggest that transition metals that are released from particle surface may be responsible for ROS generated toxicity (Dreher et al., 1997; Kodavanti et al., 1999). In addition, recent studies further suggest that inflammation observed in rats exposed to ultrafine carbon black and polystyrene particle is due to either surface area or particle number effects, in the absence of transition metals (Brown et al., 2000, 2001). However, the relationship between ROS and surface area of ultrafine particles in susceptible population is still unclear.

Reduced glutathione (GSH) functions as a coenzyme in detoxification of xenobiotics and carcinogens, and as an antioxidant (Meister and Anderson, 1983). Depletion of GSH has been used as indicator of oxidative stress following PM exposure (MacNee et al., 1997). 8-hydroxydeoxyguanosine (8-OHdG) is one of the most abundant oxidative DNA adducts, a specific DNA damage which may initiate carcinogensis. It is mutagenic in nature and can cause G to T transversion (Loft and Poulsen, 1996). Exposure to urban particles have been associated with increased DNA damage (Corero et al., 2001). In this study, we investigated the effect of ultrafine polystyrene particles on inflammation and oxidative stress markers in pulmonary hypertensive rats; to determine whether surface area of ultrafine polystyrene particles play an important role in ROS generation in diseased animals.

Materials and methods

A549 cell culture and exposure to polystyrene particle

The human A549 cell line was from American Type Culture Collection and cultured in a humidified 37^{o}C environment in F12-K medium supplemented with 10% fetal calf serum and 1% penicillin and streptomycin. This cell line, derived from a patient with alveolar cell carcinoma of the lung, has been used as a model of human alveolar type II cells. A549 epithelial cells were exposed to 100μg/ml of 64, 109 and 202-nm ultrafine polystyrene particles for 4 hours, respectively. Determination of cytokine IL-8 (R&D Systems, Minneapolis, MN) concentration was performed using ELISA.

Animals

Male Sprague-Dawley rats (body weight from 300 to 350g) were obtained from the National Laboratory Animal Breeding and Research Center, Taiwan. They were housed in plastic cages on Aspen chip bedding, and provided with Lab Diet 5001, water ad lib, except during the exposure. Animals were maintained on a 12-hour light/dark cycle at 22 + 1°C and 55 + 10 % relative humidity.

Development of pulmonary hypertension

The model of pulmonary hypertension rats following treatment of rats with Monocrotaline

(MCT) was used (Kodavanti et al., 1998). A single intraperitoneal injection of MCT (Sigma Chemical Co., 60mg/kg) was administered to each rat. Fourteen days after MCT treatment, polystyrene particles instillaiton was conducted.

Polystyrene particle instillation

Polystyrene microspheres in three sizes: 64, 202 and 535 nm average diameter (Polysciences, UK) were suspended at a concentration of 100 and 50 μg/ml in idd H2O. Estimated surface area of instilled dose was in Table 1. Animals were anesthetized and 100 and 50μg/ml of each particle suspension in 0.5 ml PBS was instilled into the lungs. Animals (n=4) received 0.5 ml PBS were as control group.

Bronchoalveoalr lavage

Rats were sacrificed 24h after intratracheal instillation. BAL fluid was collected by washing the airway with a phosphate-buffered saline solution (PBS, pH = 7.4, 28 ml/kg body weight) five times. Lavage fluid was used to determine the total number of cells and cell differential counts. Macrophage, lymphocyte, neutrophils, eosinophils and basophiles were counted under light microscopy (200 cells/slide). The remaining lavage fluid was used for the analysis of total protein and LDH activity. LDH activity was determined by autoanalyzer at National Taiwan University Hospital. Total protein was determined using total protein assay kit (BioRad Co.). Cytokine IL-6 protein and TNF-α were also determined using specific ELISA kit (R&D Systems, Minneapolis, MN).

Determination of total glutathione (GSH)

Quantitative colormetric determination of total glutathione of lung tissue were determined according to manufactures instructions (GSH-420, OxisResearch, U.S.A.). Briefly, lung tissues were homogenized in precipitation reagent at a ratio of 1 to 20 (w/v), then centrifuge homogenate at 3000 g for 10 minutes at 4 ℃. Upper aqueous layer was collected for assay. Internal calibrators ranged from 75 to 175μM. The absorbance was measured using a spectrophotometric plate reader at 420 nm wavelength.

Determination of plasma 8-OHdG level

Plasma samples were used for the determination of 8-OHdG levels with a competitive ELISA kit (Japan Institute for the Control of Aging, Japan). The determination range was 0.5-200 ng/ml. The 8-OHdG monoclonal antibody and plasma sample were loaded at 50 μl on a microtiter plate which has been coated with 8-OHdG, and incubated at 37℃ for 1 hour, in accordance with the instructions of the manufacturer. After washing, the antibodies that remained bound to the 8-OHdG in the sample were further bound with the horseradish peroxidase-conjugated secondary antibody. Subsequent addition of 3,3',5,5'-tetramethylbenzidine resulted in the development of color intensity proportional to the amount of antibody bound to the plate. The color reaction was terminated by stop solution (phosphoric acid) and the absorbance was measured using a spectrophotometric plate reader at 450 nm wavelength.

Statistical analysis

SAS software package, version 8, was used for statistical analysis. Students' t-test was used to analyze difference in each particle size and concentration. Type I error rate was set at 0.05 for significance.

Results

At both 100 and 50 μg/ml of IT particles caused significant increase in total cells in bronchoalveolar lavage and percentage of neutrophils with the 64-nm particles compared to the control and larger particle diameters (Fig 1, $p < 0.05$). In contrast, no significant effects were observed in 535-nm particles on total cells and percentage of neutrophils in both concentrations compared to the control.

An evident increase in bronchoalveolar lavage total protein and LDH activity in pulmonary hypertensive rats with 100μg/ml of 64-nm particles compared to the control and larger particle diameters (Fig 2, $p < 0.05$). No significant difference in size dependent effect was observed at treatment with 50μg/ml. Similar results were observed in bronchoalveolar laveage IL-6 protein and TNF-α (Fig 3).

Fig 4 showed that a significant depletion of total GSH in lung tissue in rats with 100μg/ml of 64-nm particles compared to the control groups. However, when all the GSH data were plotted against the surface area of particles instilled in the 100 and 50μg/ml dose, a decrease of GSH with increasing surface area was observed.

Fig 5 illustrated that all the 8-OHdG data plotted against the surface area of particles. Although the dose response relationship is not as a straight line, plasma 8-OHdG increased with the increasing surface area of particles instilled dose.

Cytokine level of A549 cell after polystyrene particle exposure was illustrated in Fig 6. We observed that ultrafine polystyrene particles induced higher levels of cytokine IL-8 as compared to controls.

Discussion

The results showed that instillation with 100 and 50μg/ml of ultrafine polystyrene particle can induce increased inflammation compared to fine polystyrene particle at same mass concentration in pulmonary hypertensive rats. We also observed that ultrafine polystyrene demonstrate significant higher proinflammatory cytokine IL-6 and TNF-α as compared to fine particles. Furthermore, the result revealed that the depletion of total GSH and formation of plasma 8-OHdG were associated with surface area of particles instilled.

In order to investigate the effect of particle size and surface area, polystyrene microspheres have been used as a model of particle without complications of transition metals and chemical property. Recent studies have reported that inert ultrafine polystyrene have proinflammatory activity at high concentration in normal rats (Brown et al., 2001). Our study further demonstrated that ultrafine polystyrene can cause marked inflammation and lung injury at much lower dose in susceptible diseased animals.

Ultrafine particles have been shown to generate free radicals and induce oxidative stress (Donaldson et al., 1996; Stone et al., 1998; Brown et al., 2001). It is generally accepted that ultrafine particles cause inflammation through a cascade of interactions via oxidative stress or interactions with surface receptors on alveolar macrophages or epithelium cell to activate signal transcription (Churg A., 1996; Donladon et el., 2002). Furthermore, this study showed that the difference in oxidative stress was attributed to the surface area of the ultrafine particles. In addition to direct depletion of antioxidant GSH, the formation of 8-OHdG has been shown to increase with surface area instilled. A recent epidemiology study suggested that long-term exposure to combustion-related particle is an important risk factor for lung cancer mortality (Pope et al., 2002). A genotoxic effect of air particulate matters is also observed in an in vitro study (Carero et al., 2001). It is believed that surface properties of particulate matter play a critical role in its ability to form ROS, which are implicated in DNA damage and carcinogenesis. ROS such as hydrogen peroxide, superoxide anions and hydroxy radicals can directly or indirectly damage cellular DNA or protein. Among these ROS, the hydroxyl radicals are generally assumed to be the critical ROS that directly attack DNA. DNA damage caused by ROS includes DNA strand breaks (Hartwig et al., 2002) and base modifications, such as 8-OHdG (Lawrence, 2002). Our results provide evidence that surface area instilled of ultraifne particles are significant associated with 8-OHdG, therefore, 8-OHdG may applied as an oxidative stress marker of ultrafine particle exposure.

In conclusion, we found inert ultrafine polystyrene may cause inflammatory effects on disease animals, and the formation of ROS was associated with instilled surface area dose. The exact mechanism warrants further study.

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	Dose (μg/ml) Surface area($cm^{2)}$	
Diameter (nm)	100 μg	50 μg
64	89.3cm^2	49.7cm^2
202	28.3cm^2	14.2cm^2
535	10.7cm^2	5.4cm^2

Table 1
Corresponding instillation surface area dose of polystyrene particles.

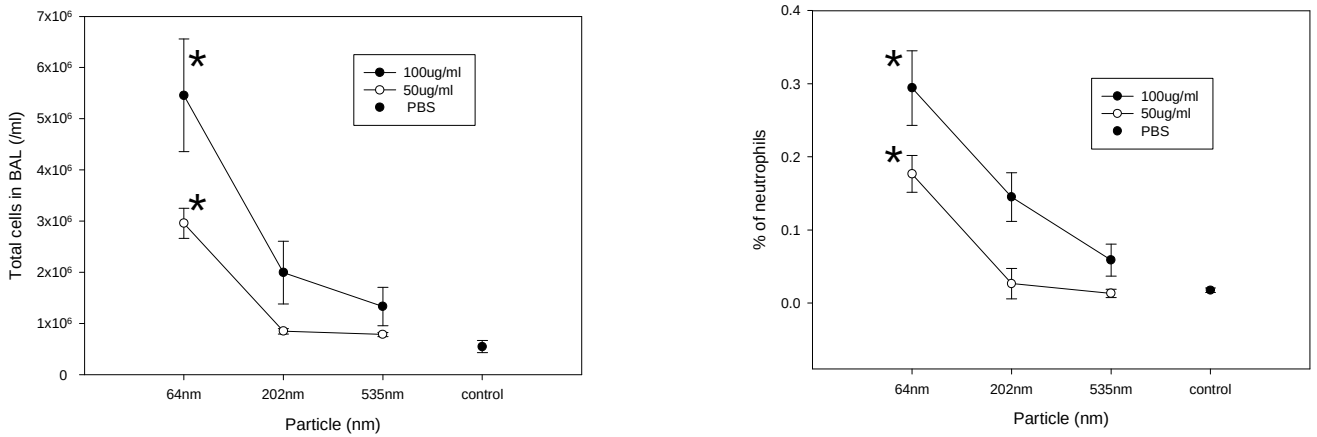

Fig 1
Total cells and percentage of neutrophils in bronchoalveolar lavage in pulmonary hypertensive rats after instillation of 100 and 50 μg/ml of polystyrene particles.
(* $p < 0.05$)

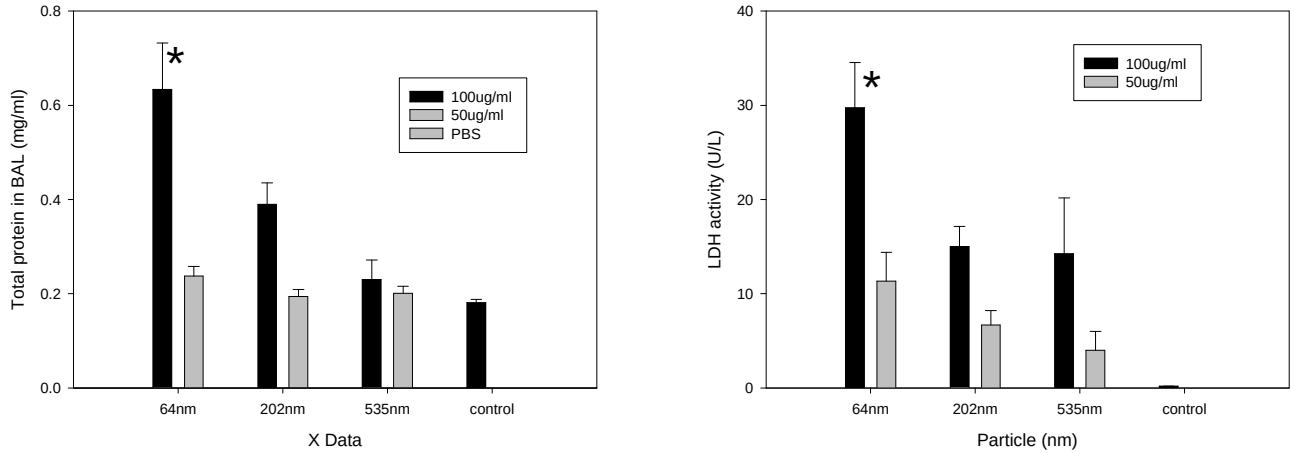

Fig 2
Total protein and LDH activity in bronchoalveolar lavage in pulmonary hypertensive rats after instillation of 100 and 50 μg/ml of polystyrene particles.
(* $p < 0.05$)

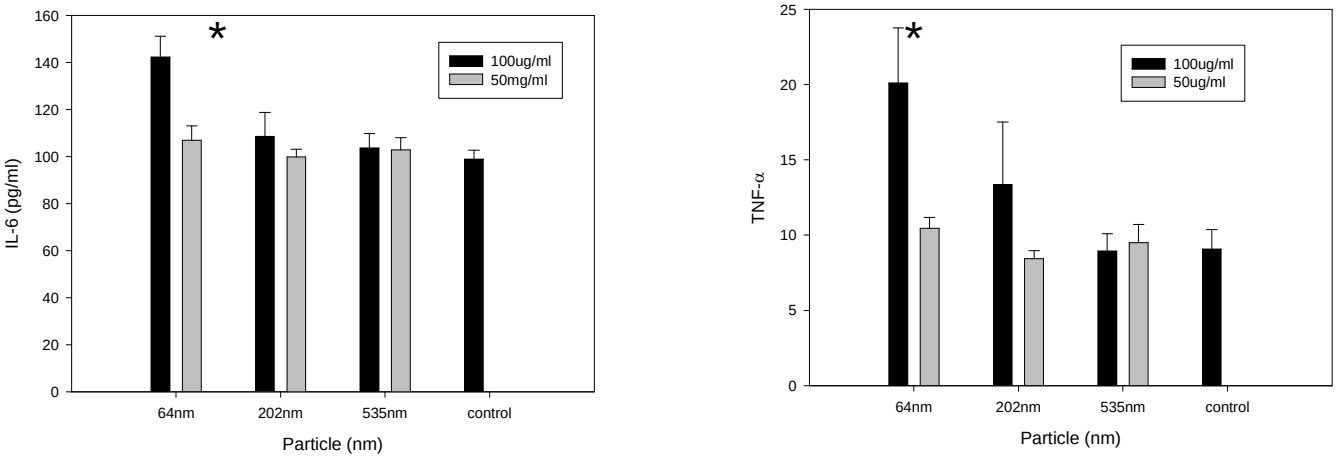

Fig 3
Proinflammatory cytokine IL-6 and TNF-α in bronchoalveolar lavage in pulmonary hypertensive rats after instillation of 100 and 50 μg/ml of polystyrene particles .
(* $p < 0.05$)

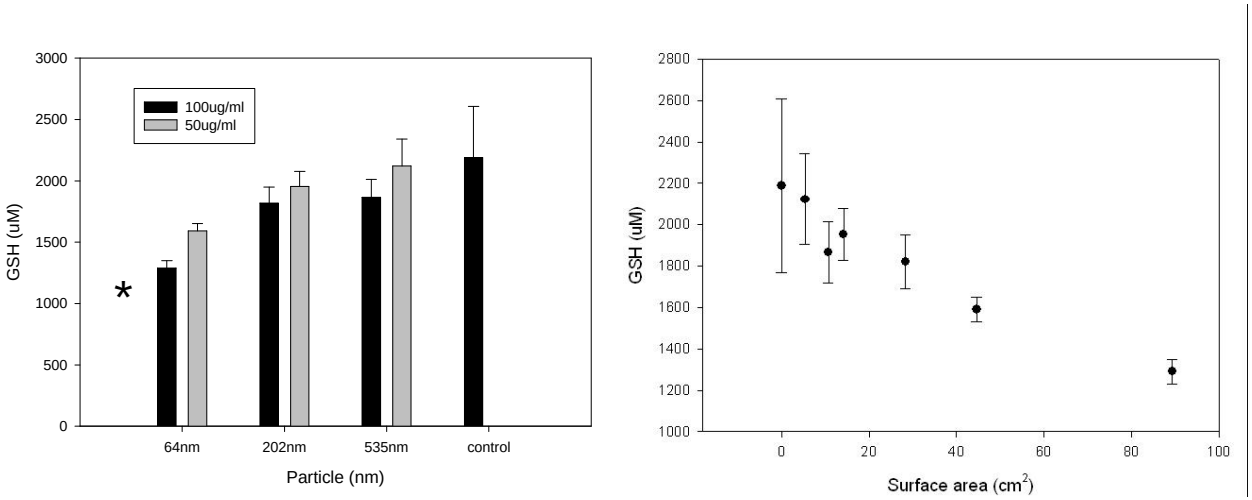

Fig 4
Total GSH in lung tissue after instillation of 100 and 50 μg/ml of polystyrene particles in pulmonary hypertensive rats, and replotted against instillation surface area.

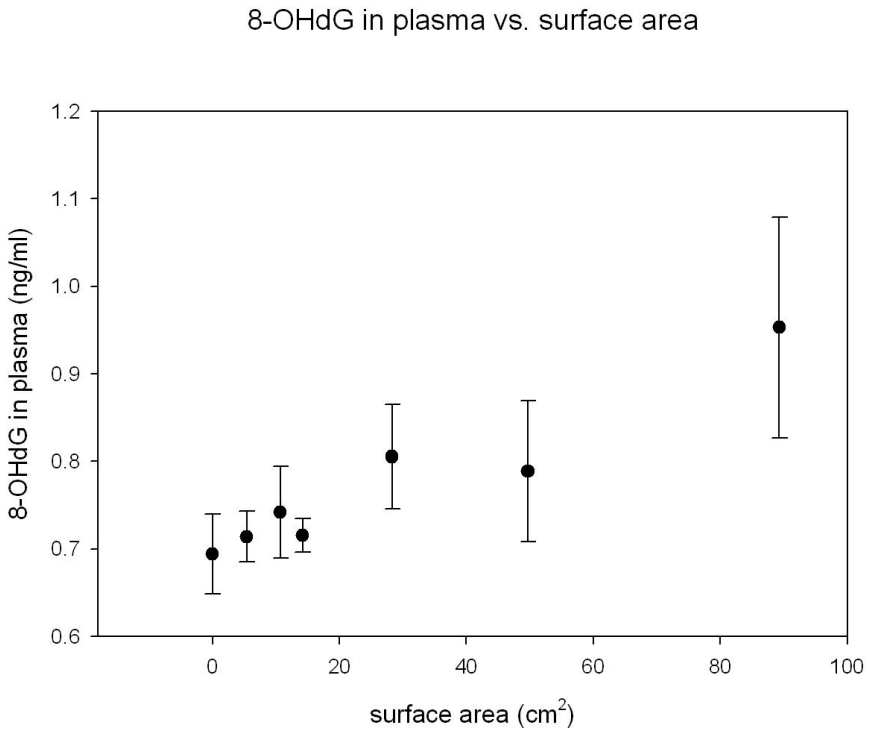

Fig 5 Mean plasma 8-OHdG response to polystyrene particles, plotted against instilled surface area dose.

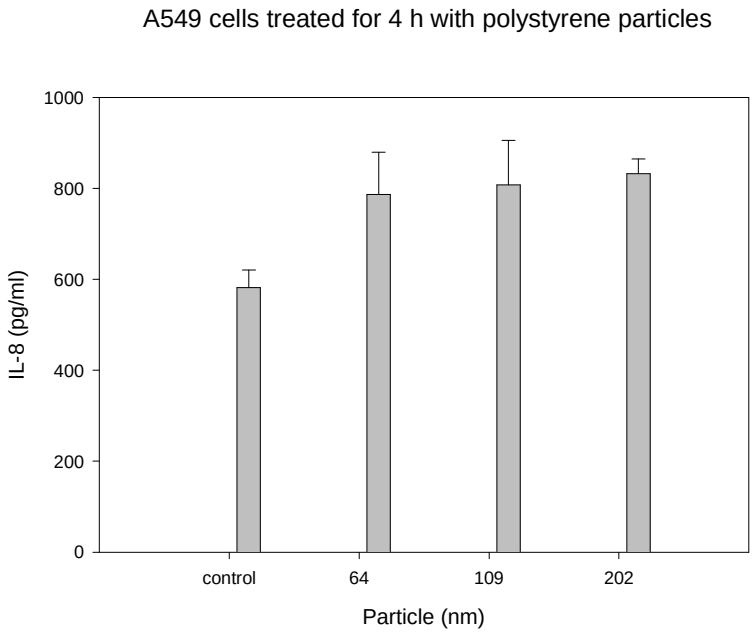

Fig 6
Cytokine level of A549 cells after exposure to 100μg/ml of 64, 109 and 202-nm ultrafine polystyrene particles for 4 hours.

奈米微粒與健康風險研究-子計畫五：疾病動物模式奈米微粒毒性探討(II)(1/2)

計畫編號：NSC 92-2621-Z-002-014
執行期限：2003 年 8 月 1 日至 2004 年 7 月 31 日
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中文摘要

近年來，許多流行病學研究指出，空氣污染中氣懸微粒的增加，與呼吸系統疾病和心血管疾病死亡率增加有關，這些死亡大都發生於已經有心肺疾病的個人，然而確實致病機轉仍不清楚。流行病學研究亦指出，長期暴露於懸浮微粒會增加肺癌死亡的風險。相關研究指出，奈米微粒(ultrafine particle, <0.1μm)可能是呼吸與心血管疾病死亡率及致病率增加的重要原因之一。根據微粒成分與毒性研究顯示，相同重量下，同材質的奈米微粒毒性較粗微粒高，可能與奈米微粒的數目及總表面積皆較粗微粒高出許多，引起氧化壓力相對較大有關；此外，奈米微粒在肺泡具有高沈積率，亦具有穿透肺泡間質，經血液循環通透至身體其他器官之特性，因此奈米微粒的暴露在健康風險上扮演極重要之角色。近年，已有一些研究針對奈米微粒與細胞毒性的關係進行探討，主要著重在奈米微粒造成的發炎反應，但是對於奈米微粒引起的呼吸道變化及心血管系統效應之間的關係並不清楚。本研究目的為：(1)探討肺部上皮內襯液體對於奈米微粒引起細胞氧化傷害的影響，評估奈米微粒與細胞反應後產生之 ROS 及評估細胞 DNA 單股斷裂情形。(2)以糖尿病大鼠模式進行奈米微粒毒性試驗，以氣管灌注方式進行 125 及 500μg/ml 奈米碳黑暴露，觀察其肺部發炎及周邊血液發炎反應、內皮細胞功能標記之變化。研究結果顯示，在非細胞系統中，奈米碳黑產生的 ROS 有隨暴露濃度及暴露時間增加呈現上升的趨勢。碳黑微粒在添加 ELF 的環境下能顯著降低 ROS 產生，同時，ELF 亦能減少 DNA 單股斷裂的情形。在糖尿病大鼠模式中，我們發現暴露於奈米碳黑導致顯著的肺部發炎及傷害反應，奈米碳黑在糖尿病大鼠造成顯著的周邊發炎反應增加，血管內皮素升高及血液一氧化氮降低，奈米微粒暴露可能與糖尿病有共同的的病理生理作用途徑，造成心血管疾病增加的風險。

關鍵詞：奈米微粒、糖尿病大鼠、肺部上皮內襯液體、 反應性氧化物種、心肺疾病

Abstract

Epidemiologic studies have shown consistent associations between the exposure to particulate air pollution and acute increase in morbidity and mortality, especially for susceptible subjects with pre-existing respiratory and cardiovascular disease. Furthermore, it has been reported that long-term exposure to combustion-related fine particles may be associated with lung cancer mortality. However, the exact mechanism remains unclear. It is believed that ultrafine particles may have a greater inflammatory effect than larger particles at the same mass concentration because of larger surface area and oxidative stress. In addition, ultrafine particles deposited in alveolar region may translocate into other organs. Thus, ambient ultrafine particles play critical role in health risk assessment. However, the role of ultrafine particles on cardiopulmonary events is not clear.

The goal of this study was: (1) to investigate the effect of epithelium lining fluids (ELF) on ultrafine particle-induced ROS generation. Ultrafine carbon black induced-ROS generation and DNA single strand breaks were evaluated. (2) to evaluate the effect of ultrafine carbon black on lung inflammation, systemic inflammation and endothelial dysfunction in STZ-diabetic rats. Our results revealed that in cell free system, the amounts of ROS increased with exposure concentration and exposure time. ELF significantly decreased ROS 90% as compared to culture medium after ufCB exposure. Furthermore, ELF also decreased DNA single-strand breakage after ufCB exposure. In diseased animal study, we found ufCB caused significant increase in pulmonary inflammation. We also observed significant alteration in systemic inflammation and endothelial dysfunction in diabetic rats exposed to ufCB. We suggest that ufCB

and diabetes may share the common pathway which may be related to cardiovascular events.

Keywords: ultrafine particles, diabetic rats, epithelium lining fluid, reactive oxygen species, cardiopulmonary disease

Introduction

Epidemiologic studies have shown consistent associations between the exposure to particulate air pollution and acute increase in morbidity and mortality, especially for susceptible subjects with pre-existing respiratory and cardiovascular disease (Pope and Dockery 1999; Samet et al., 2000; Pope et al., 2002). These epidemiologic studies also document that particles with a diameter below 2.5μm (PM2.5) have greater adverse health outcomes as compared to particles with a diameter below 10μm (PM10). Recent studies further indicate that the ultrafine particles (< 100 nm) may have an increased toxicity relative to larger particles under the same mass concentration (Ferin et al., 1992; Oberdorster et al., 1995; 2001). Many mechanisms of ultrafine particle-induced toxicity have been proposed, including greater number concentration, larger surface area and transition metals on particles (Oberdorster, 2001).

Since ultrafine particles have greater surface area compared to fine or coarse particles of same mass, greater reactive oxygen species (ROS) may be generated by the ultrafine particles. Ultrafine particles have been shown to generate ROS and cause oxidative stress (Stone et al., 1998). Several studies have demonstrated that ultrafine particles induce production of ROS both in vitro and in vitro (Stone et al., 1998; Wilson et al., 2002). Furthermore, various ultrafine particle have been demonstrated to generate more free radicals and ROS than fine particles (Stone et al., 1998; Wilson et al., 2000). Then, ROS may elicit transcription of proinflammatory cytokines and result in a cascade of inflammation events (Stone et al., 1998).

In order to investigate the effect of ultrafine particles on health effects, an in vitro study and an in vivo study were conducted in this report.

First, we investigated if airway lining fluid affects the ROS generated by ultrafine particle and subsequent DNA damage. Pulmonary epithelial lining fluid（ELF）consists of various antioxidants, which can protect lung cells against the oxidative damage of PM. However the exact mechanism remains unclear. The aim of this study was to determine the effect of ELF on PM-induced oxidative damage. Studies have found that susceptible subjects tend to have lower antioxidant in the peripheral blood and airway lining fluid. Animal studies also demonstrated that those deficient in antioxidants are more susceptible to PM exposure (Norwood et al., 2001). Those with deficiency in antioxidants are more likely to be affected by PM. Airway lining fluid contains antioxidants including Vitamin C and E and glutathione as well as enzymes including superoxide dismutase, glutathione peroxidase and catalase. Previous studies have shown that antioxidants in lining fluid decreased after PM exposure. Although the antioxidation effects have been demonstrated in cell free system, it is not clear if these changes also result in the reduction in the subsequent DNA damage. In this study, we investigated if airway lining fluid affects the ROS generated by ultrafine particle and subsequent DNA damage.

Second, we used diabetic rats to investigate the cardiovascular effect of ultrafine particles. The association between ambient particulate matter (PM) and cardiovascular diseases has been demonstrated in epidemiological studies (Pope and Dockery, 1999; Samet et al., 2000; Pope et al., 2002). Subjects with existing cardiovascular diseases including ischemic heart disease and congestive heart failure are found to be more susceptible to ambient PM exposure (Pope et al., 2002). Recently, some researchers have also suggested that diabetic patients are another sub-population at risk for PM-associated cardiovascular events (Goldberg et al., 2001; Zanobetti et al., 2001, 2002). Diabetes has been reported as an effect modifier of air pollution related hospital admissions for heart disease in elder person (Zanobetti et al., 2001), doubling the risk of a PM10-associated cardiovascular admission compared with non-diabetics (Zanobetti et al., 2002). Diabetes mellitus is a common disease, and has been associated with cardiovascular morbidity and mortality (Resnick et al, 2001). PM is also reported to affect cardiovascular diseases (Pope et al., 2004). However, the mechanisms through which ultrafine particle enhances the risks of cardiovascular disease in diabetics remain unclear. We hypothesize that diabetes and ultrafine particles may share common pathway and act synergistically in the development of cardiovascular diseases.

Hyperglycemia of diabetics has been associated with increased reactive oxygen species (ROS) formation (Maritim et al., 2003). Diabetic patients usually have significantly elevated concentrations of 8-OHdG in their serum (Nishikawa et al., 2003) and decreased levels of glutathione (GSH) (Dincer et al., 2002). It is proposed that increased ROS may induce inflammation in endothelium, alter endothelium function, and increase coagulability (Beckman et al.,

2002). Inflammation activity also increased in individuals with diabetes, as shown by increased levels of C-reactive protein (CRP) (Jager et al., 1999; Schalkwijk et al., 1999), interleukin-6 (IL-6)(Schram et al., 2003), and tumor necrosis factor (TNF-α) (Lechleitner et al., 2000). Hyperglycemia also inhibits the production of nitric oxide (NO) by blocking eNOS synthase activation and increasing the production of ROS in endothelial and vascular smooth muscle cells (De Vriese et al., 2000). In addition to reducing concentration of NO, diabetes increases the production of vasoconstrictors, most importantly, endothelin-1 (ET-1). Reports on endothelial dysfunction in patients with diabetes have been widely studied, including increased ET-1 and decreased NO (Haak et al., 1992; Williams et al., 1996).

The exact mechanisms through which ambient PM causes cardiovascular diseases remain unclear. PM exposure is associated with increased generation of ROS (Tao et al., 2003). It is proposed that PM may increase the oxidative stress related to cardiovascular disease in peripheral blood (Sorensen et al., 2003). PM exposure is also associated with elevated levels of C-reactive protein (Peters et al., 2001; Pope et al., 2003), enhanced production of proinflammatory cytokines (Ghio and Devlin 2001; Seaton et al., 1999; Schwartz 2001; Peters et al., 2001) and increased blood viscosity (Seaton et al., 1995; Peters et al., 1997) in epidemiological studies. In animal studies, the association between PM exposure and increased ET-1 has also been reported (Bouthillier et al., 1998; Vincent et al., 2001). It appears that PM and diabetes share common pathway in the development of cardiovascular diseases. Thus, we hypothesize exposure to PM may potentiate the cardiovascular diseases of diabetes through the enhanced production of oxidative stress and endothelial dysfunction.

In order to test the effects of ambient particles on diabetics, we exposed streptozotocin (STZ)-induced diabetic rats to PM. Streptozotocin is a metabolite of the soil organism streptomyces achromogenes and was first reported to be diabetogenic in studies of dogs and rats in 1963 (Bell et al., 1983). Diabetes is caused by a direct toxic effect of streptozotocin on the pancreatic beta cell. After the administration of streptozotocin, there is a characteristic increase in blood glucose, which is maintained at the level of 400mg/dL or greater. This diabetic animal model has been used in many studies of diabetes pathophysiology for years (Vural et al., 2002; Ryu et al., 2003; Zang et al., 2003)

Material and Methods

In vitro study

Chemical reagents

Albumin (BSA), lysozyme (chicken egg white), apotransferrin (human), glutathione (GSH), uric acid (UA), α-tocopherol (AT), ascorbic acid (AA), catalase (CAT, bovine liver), superoxide dismutase (SOD, in bovine erythrocytes), glutathione peroxidase (GPx, bovine erythrocytes), Phosphatidylcholine (egg)，2,7-dichlorofluorescin diacetate（DCFH-DA）were obtained from Sigma Chemical (St. Louis, MO). All chemicals were reagent grade or of higher purity.

A549 cell culture

The human A549 cell line was from American Type Culture Collection and cultured in a humidified 37oC environment in F12-K medium supplemented with 10% fetal calf serum and 1% penicillin and streptomycin. This cell line, derived from a patient with alveolar cell carcinoma of the lung, has been used as a model of human alveolar type II cells. A549 cells were cultured in six well transwell.

Particles preparation

The particles used in this study was ultrafine carbon black particles (14 nm, Degussa, Printex 90), suspended in culture medium in 50 and 150 μg/ml.

Preparation of ELF

ELF was formulated based on method of Guobin (2001) and Andre (1990). The components in ELF were listed in Table 1. Apotransferrin was used as a surrogate for lactoferrin because it has similar iron-binding qualities yet is more readily available. The following is the procedure for preparation of 100 ml of complete ELF. 1.0 ml of AT in chloroform (0.1 mg/ml) was added to a 100ml glass tube that contained 40ml idd water. The mixture was evaporated under nitrogen at room temperature. Next, 50 ml of medium were added, and the mixture was ultrasonicated in a water-ice bath., a solution of proteins (740 mg of albumin, 240 mg of lysozyme, and 20 mg of apotransferrin in 30 ml of medium) was added to this solution slowly. Finally, UA (0.5 mg/ml, 5.0 ml), AA (5 mg/ml, 1.0 ml), CAT(4 U/ml), SOD (40U/ml), GPx (0.05U/ml) and GSH (5 mg/ml, 1.0 ml) were added, and RPMI1640 was added to a final volume of 100 ml. The ELF was adjusted to pH 7.4 using NaOH (0.2 M) and H3PO4 (0.2 M).The ELFs were stored at -80°C.

Determination of ROS generation

Generation of ROS in the exposed solution of cell and free system was determined using the probe DCFH-DA（2,7-dichlorofluorescin diacetate）. The

diacetate form of the probe is both lipid soluble and nonfluorescent. Incubation of DCFH-DA with serum containing medium results in cleavage of the diacetate group by esterase enzymes to yield a relatively lipid insoluble, nonfluorescent probe (DCFH). Subsequent oxidation of DCFH by ROS yields the highly fluorescent compound 2,7-dichlorofluorescein (DCF), which can be quantified by microplate reader with excitation wavelength at 485nm（bandwidth 20nm）and an emission wavelength at 530 nm（bandwidth 25nm） DCFH-DA (final concentration 20M) was incubated for 15 min at 37°C in cell culture medium (10% FBS) in order to cleave the DCFH-DA precursor to DCFH. Suspensions of particles were prepared in RPMI1640 medium, mixed by vortexing, and then sonicated for 10 min. After exposure of the DCFH to the particles for 30 min(37℃), DCF fluorescence intensity was determined by Cytofluor 2300 microplate reader (Millipore, Bedford, MA, USA).

Determination of DNA single strand breaks by comet assay

Quantities of 20 μl of A549 suspensions (105 cells/ml) were mixed with 1 ml 1.5% low melting agarose and gelled to frost slides pretreated with 0.1 % normal melting agarose on ice. After gelling, the slides were immersed in lysis buffer then washed with PBS. They were then placed in alkaline buffer for electrophoresisat 21 V and 190 mA for 20 min. Slides were rinsed and stained in sybr green. Individual cells were examined under microscope with photomicrographic system. All slides were coded and read blindly. The images were then recorded for further image analysis.

A proprietary image processing software, which has been used in previous studies (Ma et al., 1996) was applied to calculate the distribution of DNA on the agarose. Images of 100 randomly selected cells from each set of experiment were analyzed under a fluorescence microscope adapted with an excitation of 515-560 nm and a barrier filter of 590 nm. Parameters used in the analysis were tail length (%), tail intensity (%) and tail moment. Tail length (%) was defined as the propertion of tail length in whole comet length, tail intensity (%) was defined as the proportion of tail intensity in total comet intensity, and tail moment was defined as the product of tail length and tail intensity. We used % of the tail length and intensity because of their ease in measurement (Ma et al., 1996).

In vivo studies

Particles preparation

The particles used in this study was ultrafine carbon black particles (14 nm, Degussa, Printex 90), suspended in culture medium in 125 and 500 μg/ml

Diabetic animals and intratracheal instillation of ufCB

Male Sprague-Dawley rats, weighing 200~250 g, were obtained from the National Laboratory Animal Breeding and Research Center, Taiwan. They were housed in plastic cages on Aspen chip bedding, and provided with Lab Diet 5001, water ad lib, except during the exposure. Animals were maintained on a 12-hour light/dark cycle at 22 + 1°C and 55 + 10 % relative humidity. A single intraperitoneal (IP) injection of streptozotocin (STZ, Sigma Chemical Co., 60mg/kg body weight, dissolved in citric acid buffer, pH 4.5) was administered to eight animals to induce diabetes (Bell et al., 1983). Rats were randomly divided into ultrafine carbon black exposure groups (125 and 500 μg/ml) and control group (PBS). All protocols used in this experiment were approved by National Taiwan University's animal care and use committee.

Bronchoalveolar lavage analysis

Rats were sacrificed 24h after intratracheal instillation. BAL fluid was collected by washing the airway with a phosphate-buffered saline solution (PBS, pH = 7.4, 28 ml/kg body weight) five times. Lavage fluid was used to determine the total number of cells and cell differential counts. Macrophage, lymphocyte, neutrophils, eosinophils and basophiles were counted under light microscopy (200 cells/slide). The remaining lavage fluid was used for the analysis of total protein and LDH activity. LDH activity was determined by autoanalyzer at National Taiwan University Hospital. Total protein was determined using total protein assay kit (BioRad Co.)

Determination of blood glucose, oxidative stress, inflammation and endothelial dysfunction

A total of 15 ml whole blood was recovered from aorta. Immediately, 1 ml of whole blood was collected in citrate tube for blood glucose analysis using autoanalyzer (Glucometer 3, blood glucose meter; Miles Inc.) at National Taiwan University Hospital. 10ml whole blood was collected in ethylenediamine tetraacetic acid (EDTA) tubes. After centrifuged at 1200 rpm for 10 minutes, plasma aliquots were stored at -80℃ until analysis. The remaining 4 ml whole blood was collected in glass tube. Circulating inflammation markers were represented by cytokine IL-6 and TNF-α. Plasma levels of IL-6 and TNF-α were measured using ELISA kits (R&D Systems, U.S.A). Plasma ET-1 and NO were used to represent endothelial function. ET-1 was measured by ELISA (R&D Systems) with human ET-1 as standard (Bouthillier et al., 1998). Nitric oxide synthase assay kit with the Griess

reagent (CALBIOCHEM Inc., U.S.A.) was used to determine plasma NO. The plasma concentration of NO was calculated as the sum of nitrite (NO2-) and nitrate (NO3-) concentrations.

Statistical analysis

SAS software package, version 8, was used for statistical analysis.

Results and Discussion

In vitro studies

Ultrafine carbon black (UfCB) induced a significant increase in DCF fluorescence at 50 and 150μg/ml as compared to control (Figure 1, $p < 0.05$). Similarly, A549 cells exposed to 50μg/ml or 150μg/ml, and then incubated with DCFH for 30 min, exhibited a significant greater fluorescence than control (Figure 1, $p<0.05$). In cell-free system and with A549, ELF addition result in significant decrease in DCF fluorescence induced by UfCB (Figure 1). Further, increasing oxidative DNA damage was observed with increased UfCB concentration (figure 2). Again, with ELF, the oxidative DNA damage was reduced. Our results found that UfCB could induce ROS in cell free system. It appears that surface of UfCB may react with water and induced ROS, which can cause DNA damage in A549 cells. Because ELF can decrease total ROS and DNA damage induced by UfCB, this further supports that ROS plays an important role in ultrafine particles related toxicity. However, the exact mechanisms through which ultrafine particles induce toxicity needs further study.

In vivo studies

Characteristics of study animals were described in Table 2. Body weight of diabetic rats was lower than that of non-diabetic rats (510.5 g vs. 580.2 g). The mean plasma glucose level was 150.5 mg/dl in non-diabetic rats, and 350.5 mg/dl in diabetic rats ($p < 0.05$).

Diabetes had no effect on total cells, percentage of neutrophils, total protein and LDH activity in BAL (Table 3). In plasma analysis, diabetic rats demonstrated significantly greater cytokine IL-6 as compared with non-diabetic rats ($p <0.05$). Furthermore, diabetic rats had significantly increased level of plasma ET-1 ($p < 0.05$), and decreased level of plasma NO ($p < 0.05$).

In non-diabetic rats, ufCB caused significant increases in total cells and proportion of neutrophils in bronchoalveolar lavage (Table 3, $p < 0.05$). Elevated total protein and LDH activity in bronchoalveolar lavage were also observed after ufCB exposure ($p<0.05$). Plasma IL-6 level also showed significant increase after ufCB exposure (Table 4, $p < 0.05$). Furthermore, ufCB exposure caused a significant reduction of plasma NO ($p<0.05$). However, there was no significant ufCB effect on plasma ET-1 in non-diabetic rats.

In STZ-diabetic rats, significant increases in pulmonary inflammation and injury markers were observed after ufCB exposure (Table 3, $p<0.05$). Plasma IL-6 and cytokine TNF-α significantly increased in diabetic rats after exposure to ufCB (Table 4, $p < 0.05$). In assessing endothelial function, we found a significant elevation of plasma ET-1 and a significant decrease in plasma NO after exposure to ufCB ($p < 0.05$).

Similar findings were observed in our previous study in which, CAPs enhanced the endothelial dysfunction in diabetic rats as compared to non-diabetic rats. In this study, we further observed that ultrafine particles have similar effects. It is not clear whether ultrafine particles penetrate into circulation then directly cause endothelial dysfunction, or indirectly affect endothelium as a result of lung inflammation. Thus, more studies are needed to investigate the underlying mechanisms. We conclude that ufCB exposure may enhance the risk of cardiovascular diseases through the synergistic interaction between ufCB and diabetes in endothelium.

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Table 1 components and concentrations of ELFs

Constituents of synthetic lung epithelial ling fluid	
components	concentration
Serum albumin, mg/ml	7.4
Phosphatidylcholine (egg), mg/ml	10
Lysozyme, mg/ml	2.5
Apotransferrin , mg/ml	0.2
Ascorbic acid (AA), g/ml	50
Glutathione reduced (GSH), g/ml	50
Uric acid (UA), g/ml	25
a-tocopherol (AT), g/ml	1
Catalase, U/ml	4
Glutathione peroxidase (GPx) U/ml	0.05
Superoxide dismutase (SOD), U/ml	40

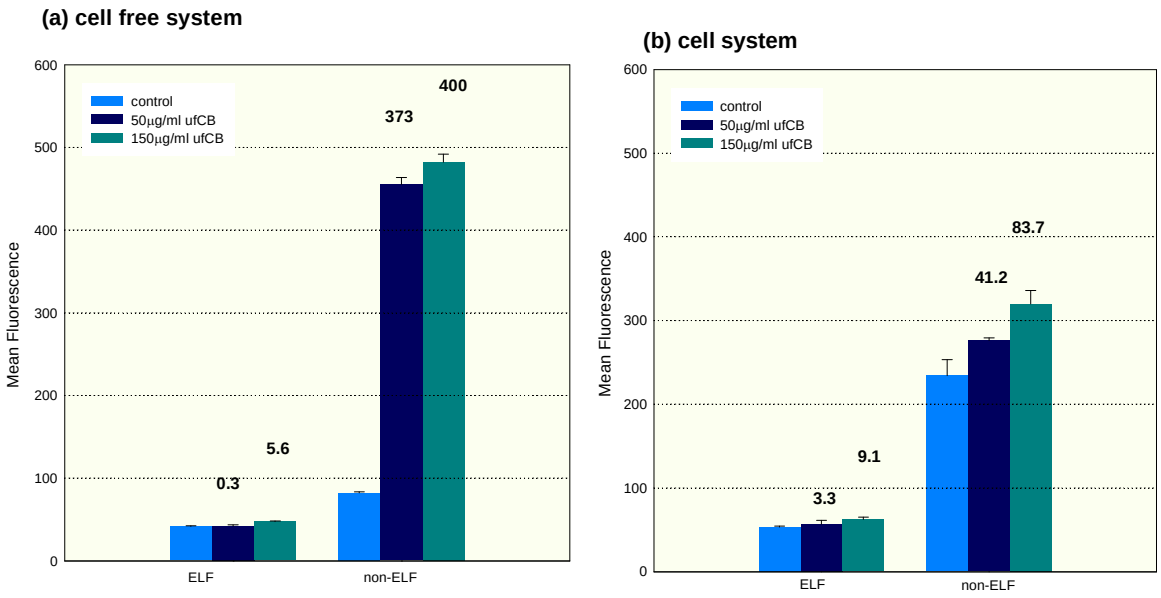

Figure 1
Effect of ultrafine carbon black on the oxidation of DCFH to DCF in (a) a cell free system, (b) a A549 cells system with or without ELF. * $p<0.05$ compared to control; **$p<0.05$ compared to 50μg/ml.

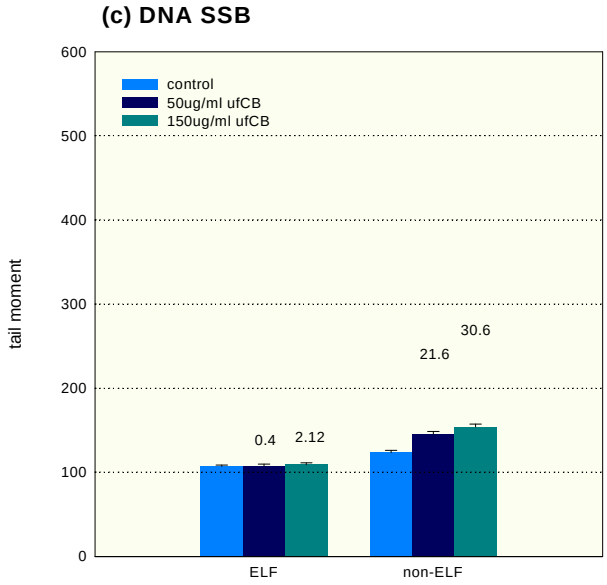

Figure 2
Effect of ultrafine carbon black on single strand breaks assessed by the comet assay.

Table 2 Basic characteristics of experimental animals

	Non-diabetic rats (n=13)	STZ-diabetic rats (n=12)
Body weight (g)	510.5±8.3	580.2±29.7*
Blood glucose (mg/dL)	150.5±9.7	350.5±9.7*

* Mean + standard deviation, * $p<0.05$ as compared to non-diabetic rats

Table 3 Cell number, cell differential and pulmonary injury markers in BAL

	Non-diabetic rats			Diabetic rats		
	Saline control	UfCB 125 μg/ml	UfCB 500 μg/ml	Saline control	UfCB 125 μg/ml	UfCB 500 μg/ml
N	4	5	4	4	4	4
Total cell (x 10^4cell)	6.3(1.1)	6.5 (1.2)	8.1 (1.1) *	5.9 (1.0)	6.2 (1.5)	7.9 (1.9)
Marcrophage %	90 (1.2)	70 (3.4)	52.1 (4.5)	92 (0.7)	72.3 (5.4)	50.3 (7.4)
Lymphocyte %	2.5 (0.2)	5 (1.3)	5.3 (1.8)	2.6 (1.8)	6.2 (1.4)	4.6(1.8)
Neutrophil %	3.5 (1.2)	21 (2.3)*	40.4 (6.7)*, **	3.4 (0.2)	19.6(3.9)*	42.1(3.9)*, **
Eosin %	1.5 (0.7)	2.4 (1.3)	1.6 (1.3)	1,3 (0.6)	1.5 (0.7)	1.7 (1.0)
Baso %	1.0 (0.3)	1.6 (0.4)	0.6 (0.2)	0.7 (0.3)	0.4 (0.1)	1.3 (0.2)
Total protein (μg/ml)	390 (43.5)	420 (39.0)	620 (15.2)*, **	402(29.4)	415 (25)	654 (10.2)*, **
LDH activity (U/ml)	140.5 (5.5)	605.0 (20.4)*	1200 (10.5)*, **	210.2 (25.4)	548.0 (22.5)*	1350 (2.5)*, **

* $p<0.05$ as compared to each saline control
** $p<0.05$ as compared to 125 μg/ml exposure group

Table 4
Systemic inflammatory and endothelial dysfunction markers in plasma

	Non-diabetic rats			Diabetic rats		
	Saline control	125 μg/ml	500 μg/ml	Saline control	125 μg/ml	500 μg/ml
N	4	5	4	4	4	4
Systemic inflmmation						
Plasma IL-6 (pg/ml)	30.5 (8.1)	39.8 (4.5)	50.2 (2.7) *, **	41.2 (5.2)	50.8 (4.7)	65.0 (7.5) *

Plasma TNF-α (pg/ml)	4.6 (2.5)	4.3 (3.2)	6.8 (1.1)	5.1 (1.2)	4.9 (1.5)	8.2 (0.7) *, **
Endothelial dysfunction						
Plasma ET-1 (pg/ml)	1.3 (0.5)	1.9 (0.4)	2.0 (0.8)	1.9 (0.3)	2.1 (0.7)	2.9 (0.1)*
Plasma NO (μM)	89.4 (4.7)	90.5 (1.8)	85.8 (5.0)	80.5 (2.0)	75.2 (3.9)	72.2 (1.5)*

* $p<0.05$ as compared to each saline control

** $p<0.05$ as compared to 125 μg/ml exposure group

奈米微粒與健康風險研究-子計畫五：疾病動物模式奈米微粒毒性探討(III)(2/2)

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摘要

隨著奈米科技的發展，人造奈米材料被廣泛的應用在科學、技術及醫學上。奈米材料可以使用在一些較大粒徑微粒所無法達到的特殊目的上，但奈米材料跟較大粒徑微粒相比較，可能具有更高的生物活性。最近的研究顯示人造奈米微粒造成健康危害可能與反應性氧化物種（reactive oxygen species, ROS）有關，因此本研究將探討奈米微粒 ROS 的產生與微粒表面積之相關性，並進一步以自發性高血壓大鼠（spontaneous hypertensive rat, SHR）及健康的 SD（Sprague Dawley）大鼠動物模式，探討奈米微粒的暴露與氧化壓力、肺部發炎反應之相關，同時我們也以敏感的 SHR 測試奈米碳黑暴露後，時間與肺部發炎的關係。

非細胞系統利用 15、51 及 95nm 的奈米碳黑微粒（ultrafine carbon black, ufCB），於 200、400 及 800 μg/ml 下暴露一小時，再以 2',7'-dichlorofluorescin（DCF）螢光分析法評估 ROS 的產生。動物實驗部分，分別以 SHR 及 SD 大鼠為實驗動物，以氣管灌注 15、51 及 95nm 的 ufCB 500 及 1000 μg，並以磷酸生理食鹽水（phosphate buffered saline, PBS）為控制組，暴露 24 小時後犧牲，取肺泡灌洗液分析肺部發炎指標；另外分析血清 8-hydroxy-2'-deoxyguanosine（8-OHdG）及周邊白血球 DNA 單股斷裂（DNA single-strand breaks, DNA SSB）情形。Time-course 實驗則氣管灌注生理食鹽水、500 及 1000 μg 的 ufCB，在灌注後 1、3、6 及 24 小時之後進行動物犧牲，取肺泡灌洗液分析總細胞數及白血球分類。

實驗結果顯示，在非細胞系統中，各種粒徑之 ufCB ROS 的產生，隨重量濃度增加而增加，在相同重量濃度下，粒徑越小的 CB 產生的 ROS 越高，有趣的是，總表面積與 ROS 產生有高度相關（R2＝0.83）。動物實驗部分，比較 SHR 與 SD 大鼠肺部發炎情形及血清 8-OHdG 的差異，不論是實驗動物的基本值或隨總表面積增加的趨勢，SHR 肺泡灌洗液中總細胞數及血清 8-OHdG 都比 SD 大鼠來得高。

Time-course 研究部分，結果顯示實驗動物在氣管灌注 500 及 1000 μg ufCB 後第 1、3、6 小時，肺泡灌洗液中總細胞數及嗜中性球比例有上升趨勢，但未達統計顯著差異。暴露後 24 小時後，則可觀察到顯著的發炎反應，顯示儘管在相當高劑量的暴露之下，肺部發炎反應仍然需在暴露 24 小時後才觀察的到。

總結來說，本研究發現奈米微粒能產生 ROS，而 ROS 的產生與微粒總表面積有關，動物實驗部分證明奈米微粒會增加體內 ROS 的產生及肺部發炎反應，而且在 SHR 易感性動物更加明顯。本研究指出易感性動物對於肺部發炎及氧化傷害的危險性增加，這部份具有重要的政策意涵，但需要進一步的研究去釐清其相關機制。

關鍵字：奈米微粒，反應性氧化物種，發炎反應

Abstract

Nanoparticles are increasingly used in science, technology and medicine, and they are produced for specific purposes which cannot be met by large particles and bulk material. However, recent evidences reveal that nanoparticles are likely to be highly reactive with biological systems. Recent studies indicated that artificial nanoparticals-induced health effects may be associated with the generation of reactive oxygen species (ROS). However, the exact relationship remains unclear. In order to investigate the possible mechanism, we used a cell-free system study to verify the relationship between total surface area and ROS generation. Further, we investigated the effects of nanoparticle exposure on oxidative stress and pulmonary inflammation on spontaneously hypertensive rats (SHR) and Sprague Dawley (SD) rats.

In cell-free system, ultrafine carbon black (ufCB) with average diameter of 15, 51 and 95nm were suspended in phosphate buffered saline (PBS) in 200, 400 and 800 μg/ml for 1hr. DCF (2',7'-dichlorofluorescin) assay was used to

determine the ROS generation of ufCB. In animal study, SHR and SD rats were intratracheally instillation of 15, 51 and 95nm ufCB in 500 and 1000μg, separately. Animals administrated PBS were treated as control group. Animals were sacrificed 24hr after treatment. Bronchoalveolar lavage fluid (BALF) was collected for pulmonary inflammation analysis. Serum 8-OHdG (8-hydroxy-2'-deoxyguanosine) and peripheral blood DNA single-strand breaks (DNA SSB) were determined to evaluate the effects of oxidative stress.

Our results revealed that the generation of ROS increased with the instilled mass concentration in each particle size. At the same mass concentration, smaller particle size of CB produced greater ROS. Interestingly, the generation of ROS was highly correlated with total surface area of particles (R2=0.83). In animal study, serum 8-OHdG and BALF total cells at baseline or their trends for total surface area in SHR was higher than those in SD rats.

In summary, we found nanoparticles generated ROS in cell free system and the generation of ROS was associated with total surface area of particle. In vivo study found that exposure to nanoparticles caused ROS generation and pulmonary inflammation. And the results were more prominent in SHR susceptible animals as compared to SD rats. Our study indicates that susceptible animals maybe subject to increased risk of lung inflammation and oxidative DNA damage. This may have important policy implication. However, more studies are needed to clarify the above findings.

Keywords: nanoparticle, reactive oxygen species, inflammation

背景

奈米是指粒徑小於 100nm，相同重量濃度下奈米微粒與較大粒徑的比較具有較大表面積及化學活性（Gilmour, et al., 2004; Renwick et al., 2004; Brown et al., 2000），另外奈米微粒因為粒徑小的特性，被吸入肺部後，在肺內的分佈與沈降位置與較大粒徑不同，可能有較多數量會沈積在肺部深層（肺泡）的部位，奈米微粒也可能直接穿透細胞、組織進入血液循環系統（Nemmar et al., 2002a）。

流行病學研究證據指出，大氣中微粒污染物的濃度與各種健康效應有關，包含呼吸道及心血管疾病的發生率、死亡率、住院率及致病率（Gauderman et al., 2004; Atkinson et al., 1999; Pope et al., 2002, 2004）；在動物體內及細胞實驗也發現，奈米微粒對肺部及 DNA 造成傷害與反應性含氧物種（reactive oxygen species, ROS）有關(Wilson et al., 2002; Li et al., 1999; Dick et al., 2003）。

Wilson 等人在沒有生物或細胞的非細胞系統中測量 ufCB ROS 的產生，發現有自發性 ROS 產生，ufCB 比 CB 能引起較多的 ROS（Wilson et al., 2002）。本實驗室之前所進行的研究中，初步研究也發現，在非細胞系統暴露 ufCB 的部分，ROS 隨微粒濃度增加而升高，在細胞實驗中暴露 ufCB 的部分，細胞外 ROS 的產生與 DNA 單股斷裂（DNA single-strand breaks, DNA SSB）情形會隨暴露濃度增加而升高（鄭伊玲，2004）。故本研究欲進一步以不同粒徑及濃度之奈米微粒進行非細胞系統 ROS 之量測，探討奈米微粒 ROS 的產生與微粒粒徑大小及表面積之相關性，另外以自發性高血壓大鼠（spontaneous hypertensive rat, SHR）及 SD（Sprague Dawley）大鼠暴露不同粒徑及濃度的 ufCB，探討微粒總表面積與氧化傷害及肺部發炎之相關性，並比較抗氧化能力較差的 SHR 與健康的 SD 大鼠兩種實驗動物對氧化傷害及發炎反應的差異。

材料與方法

整體實驗設計

實驗包含非細胞系統及動物實驗兩部分。非細胞系統使用 15、51 及 95nm ufCB，濃度為 200、400 及 800 μg/ml，並以 PBS 為控制組，在超音波震盪器中暴露一小時後，以 DCF 螢光分析法評估 ROS 的產生，實驗每個濃度執行 3 重複，每組實驗重複進行三次。動物實驗部分，分別用 26 隻 SHR 及 29 隻 SD 大鼠，以氣管灌注方式暴露 15、51、95nm 的 ufCB 500 及 1000 μg，並以 PBS 為控制組，每組不同粒徑及濃度使用實驗動物的數目為 3～4 隻，暴露 24 小時後犧牲，取肺泡灌洗液分析肺部發炎指標，包含總細胞數、血球分類計數、LDH 及總蛋白質；另外取實驗動物周邊血液分析 DNA 損傷情形，包括周邊白血球 DNA SSB 及血清 8-OHdG 分析，SD 大鼠並取肺泡灌洗液分析 8-OHdG 含量。

微粒

15nm 碳黑微粒由韓國德固薩公司贈與，51、95nm 碳黑微粒由台灣德固薩公司贈與。微粒表面積計算 15、51、95nm 分別為 300、30、20m2/g（資料由廠商提供），以氮吸收法（BET, Brunaure-Emmet-Teller）測得。

實驗動物

SHR 由國家實驗動物中心購得，飼養於台大醫學院實驗動物中心，維持 12 小時晝夜循環，溫

度約 21±1℃，相對濕度 55±10%，實驗數目共 26 隻，皆為雄性，購入時週齡 6～8 週，實驗時週齡 23～24 週，體重介於 320～360g 之間。SD 大鼠由樂斯科生物科技股份有限公司（20 隻）及國家實驗動物中心（9 隻）購得，同樣飼養於台大醫學院實驗動物中心，數目共 29 隻，皆為雄性，購入時週齡 6～7 週，實驗時週齡 9～10 週，樂斯科 SD 體重為 319±22g、國科會 SD 體重為 418±17g。

非細胞系統自由基分析：DCF 螢光分析法

參考 LeBel（1992）及 Wang（1999）、Catheart（1983）等人的方法並加以改進，先利用乙醇將 DCFH-DA 溶解，隨後利用 NaOH 將 DCFH-DA 裂解為不具螢光特性的 DCFH，再與 ROS 進一步反應成為具螢光特性的 DCF，利用讀盤螢光儀（Molecular Device SPECTRA MAX GEMINE XS）量測螢光強度值代表暴露溶液中 ROS 的含量。

動物實驗微粒氣管內灌注、肺泡灌洗及採血

將 15、51 及 95nm ufCB 秤取後懸浮於磷酸生理食鹽水（phosphate buffered saline，PBS），配置成濃度為 2000 及 4000 μg/ml，超音波震盪 15 分鐘。實驗動物以 Pentobarbital（50mg/kg body weight）麻醉後給予氣管灌注 0.25ml 的 PBS 及 15、51、95nm，500 及 1000 μg 高低兩組劑量的 ufCB，暴露 24 小時後犧牲進行腹主動脈採血，並以 PBS 進行肺泡灌洗。

肺泡灌洗液發炎指標分析

本研究以肺泡灌洗液中的總細胞數、血球分類計數（以嗜中性球及巨噬細胞百分比為主）、LDH 及總蛋白質等指標分析肺部發炎情形。取第一次抽出之肺泡灌洗液，離心後收集上清液量測 LDH 及總蛋白質，LDH 的量測交由台大實驗動物中心研發組代為檢驗（儀器型號為 ARKRAY SPOTCHEN SP-4410），總蛋白質以分光光度計量測分析（型號為 UV-160A），其它四次收集之肺泡灌洗液取適量進行總細胞數及血球分類計數。

周邊白血球 DNA 單股斷裂分析

以慧星分析法進行 DNA SSB 分析，研究方法參考 Singh 等人於 1988 年改良後之方法，取動物周邊血液 10 μl，與 1.5% LMP 混合後製成顯微玻片，加入 lysis buffer 等待細胞膜溶解，DNA denature 後置於電泳槽內，加入鹼性的 running buffer，以 21 伏特跑電泳 20 分鐘，隨後利用 sybr green 染色後置於螢光顯微鏡下分析。細胞在跑電泳的過程中會增加其 DNA 損傷的表現，於中心核後產生類似尾巴的 DNA 碎片，藉由評估影像尾巴長度所佔的百分比（% of tail length）及影像尾巴亮度所佔的百分比（% of tail intensity）及以上兩個指標的乘積（tail movement）來表示 DNA 損傷的程度，本實驗並以 A549 細胞做為內標進行校正。

血清 8-OHdG 含量分析

取大鼠週邊血液，於試管靜置 30 分鐘，以 3000rpm 離心 10 分鐘，取出血清至少 2 cc冰於 -80℃，保持低溫送至國家衛生研究院，吳焜裕老師實驗室使用 LC-MS/MS 代為分析；實驗數據分析部分，剔除平均值±3 倍標準差外的異常值。

統計分析方法

本研究以 SPSS for windows 11.0 版及 SAS system for windows 8.0 版統計軟體進行分析，實驗數據以平均值加減標準差（mean±SD）表示，暴露組與控制組之間比較以 Student’s t-test 及 Mann-Whitney U test 分析，相關性分析採皮爾森相關分析法，線性迴歸分析利用 SAS GLM 程序分析迴歸顯著性，$P<0.05$ 視為有統計上顯著差異。

結果

非細胞系統自由基分析

在非細胞系統量測不同粒徑及濃度微粒 ROS 的產生，實驗結果如圖一，三種粒徑之 ufCB ROS 的產生都隨重量濃度增加而增加，有劑量反應關係，且在相同重量濃度下，粒徑越小 ROS 的產生越高；15nm ufCB ROS 的產生在高、中、低劑量都顯著較控制組高（$P<0.05$），ROS 增加的趨勢 15nm 比 51 及 95nm 都來得高。微粒總表面積與 ROS 的產生，實驗結果如圖二，ufCB ROS 的產生隨著總表面積增加而越高，且有高度相關（$R2=0.83$）。

動物實驗肺泡灌洗液發炎指標分析

SHR 實驗部分，26 隻實驗動物麻醉後進行肺泡灌洗，其中 20 隻可觀察到肺泡灌洗液中有少許紅血球（以細胞分類染色後之標本加以確認，在 10 x 40 倍率光學顯微鏡整個視野下少於 10 個紅血球），另外 4 隻有大量紅血球（在 10 x 40 倍率光學顯微鏡整個視野下多於 10 個紅血球），肺部出血率為 0.92，嚴重出血之 4 隻實驗動物，各肺部發炎指標皆不予納入計算。SD 大鼠實驗部分，29 隻麻醉後進行肺泡灌洗，其中 5 隻可觀察到肺泡灌洗液中有少許紅血球，另外 7 隻有大量紅血球，肺部出血率為 0.41，嚴重出血之 7 隻實驗動物，各肺部發炎指標皆不予納入分析。

比較 SHR 及 SD 大鼠暴露 ufCB 微粒總表面積與肺泡灌洗液中各發炎指標分析結果如圖三，

SHR 及 SD 大鼠總細胞數與暴露微粒總表面積有顯著相關（SHR R2＝0.52，P=0.04；SD rats R2＝0.57，P=0.01），且 SHR 隨總表面積增加的趨勢顯著高於 SD 大鼠，兩線性迴歸有統計上顯著差異（P<0.0001）；SHR 嗜中性球百分比與暴露微粒總表面積有顯著相關（R2＝0.52，P=0.02），且隨總表面積增加而升高；SHR 之 LDH 與暴露微粒總表面積也有顯著相關（R2＝0.5，P=0.01），但反而是隨總表面積增加而降低；SHR 及 SD 大鼠其它肺部發炎指標與暴露微粒總表面積則未達統計上顯著。

動物實驗周邊白血球 DNA 單股斷裂分析

比較 SHR 及 SD 大鼠暴露 ufCB 微粒總表面積與 DNA SSB 各參數指標分析結果如圖四，SHR 之 DNA SSB 各參數指標與暴露微粒總表面積皆有顯著相關（tail moment R2＝0.57，P=0.0001；％ of tail length R2＝0.5，P=0.001；％ of tail length intensity R2＝0.69，P=0.0005），且隨總表面積增加而增加；SD 大鼠 DNA SSB 各參數指標沒有隨暴露總表面積增加反倒有降低的趨勢但未達統計上顯著；兩實驗動物線性迴歸皆無統計上顯著差異。

動物實驗血清 8-OHdG 含量分析

比較 SHR 及 SD 大鼠暴露 ufCB 微粒總表面積與血清 8-OHdG 分析結果如圖五，SHR 血清 8-OHdG 與暴露微粒總表面積有顯著相關（R2＝0.64，P=0.002），且隨總表面積增加而增加；SD 大鼠血清 8-OHdG 沒有隨暴露總表面積增加反倒有降低的趨勢但未達統計上顯著；兩實驗動物線性迴歸有統計上顯著差異（P<0.0001）。

暴露後時間與發炎反應關係 time-course study

SHR 大鼠在灌注 ufCB 500、1000 μg 後第 1、3、6 小時，肺泡灌洗液中總細胞數都有上升趨勢（圖六），至第 24 小時後則有顯著發炎反應(p<0.05)。肺泡灌洗液中嗜中性球比例亦有類似趨勢(圖七)。

討論

實驗結果發現，在非細胞系統中各種粒徑之 ufCB ROS 的產生，隨重量濃度增加而增加，而在相同重量濃度下，粒徑越小的 ufCB 產生的 ROS 越高，總表面積與 ROS 產生有高度相關。各粒徑濃度 ufCB ROS 的產生如預期中有粒徑效應及劑量反應關係，且 15nm ROS 增加的趨勢較 51 與 95nm 明顯，而 51 與 95 間則沒有顯著差別，這部分可能影響實驗結果的原因包括微粒表面積的差異、微粒的聚集、微粒製造過程及特性不同等。

微粒表面積方面，本研究中微粒表面積 15、51、95nm 分別為 300、30、20m2/g，15nm ufCB 為 51 及 95nm 的 10～15 倍，但 51nm 與 95nm 只差了 1.5 倍，可能因為表面積的差異，造成 15nm 所產生的 ROS 增加趨勢比 51、95nm 來得高，而 51nm 與 95nm 間表面積相差不大，故 ROS 的增加在 51 及 95nm 兩粒徑間沒有明顯的差異。Wilson 的研究也發現在非細胞系統 ufCB 能自發性的產生 ROS，而 CB 則無法產生 ROS，認為與 ufCB 具有較大表面積及具有可吸附的特性有關（Wilson et al., 2002）。

微粒的聚集方面，Wilson 以 DCF 螢光分析法分析 14nm ufCB 在非細胞系統所產生的 ROS，結果發現濃度在 15.5～124 μg/ml 間 ufCB 所產生的 ROS 有劑量反應關係，但在較高濃度部分 ROS 的產生反而降低（Wilson et al., 2002）。本研究初期的實驗也遭遇相類似情形，ufCB 在較高濃度所量測到的螢光值反而逐漸下降，這部分推測與 ufCB 為不可溶物質會影響 DCF 螢光分析法的量測有關。以 DCF 螢光分析法測量非細胞系統 ROS，不可溶之奈米微粒在高濃度下會阻擋光線穿透而影響螢光值測定；本研究以 220nm 過濾頭（cameo syringe filter nylon 13MM）過濾暴露 1 小時後之奈米微粒溶液，但 220nm 過濾頭僅能過濾聚集後形成大顆粒的微粒，低於 220nm 之微粒仍可能通過過濾頭影響螢光值之測定，故在此實驗中粒徑越小量測到的 ROS 可能被低估得越嚴重。微粒在溶液中聚集的現象在其它研究中也有提到，Brown 的研究中以穿透式電子顯微鏡觀察 14nm ufCB，發現微粒聚集後可達長 205nm 寬 135nm（Brown et al., 2000）；Gilmour 的研究中也指出，在電子顯微鏡下觀察 14nm ufCB 及 260nm CB，平均粒徑分別為 114、268nm，ufCB 聚集的情形是 CB 的 10 倍（Gilmour et al., 2004）；微粒聚集的問題在近年來的國際奈米科技研討會中也被提出來熱烈討論，但目前尚未得到良好的解決方法。

微粒生產製造過程及特性不同也可能影響微粒聚集及 ROS 的產生。實驗中所用 15、51 及 95nm 之 ufCB 皆為德固薩公司所提供，但微粒特性仍有一些不同，包括 15 及 51nm 是以 Furnace 法製造，而 95nm 是以 Lamp 法製造；在 950℃下揮發物百分比 15nm 為 1.5％，51nm 為 0.7％，（95nm 廠商無提供資料）；含灰量百分比 51nm 為 0.1％、95nm 為 0.02％（15nm 廠商無提供資料）；15、51、95nm pH 值分別為 8、9、7.5；吸油量為分別為 60、96、117ml/100g，吸油量高表面可能帶有較多 COOH 官能基，pH 值會偏酸且較易分散；若將配置好之奈米碳黑溶液放置 24

小時，微粒沈澱後 15nm 溶液呈現淡黃色，51、95nm 呈清澈透明，可看出顏色上顯著差異，且在肉眼觀察下即可看出離開超音波震盪器後微粒有快速聚集、沈澱的情形。

動物實驗肺部發炎部分，比較兩實驗動物的差異性，SHR 的肺泡灌洗液總細胞數，不論是在動物基本值或隨總表面積增加的趨勢上都較 SD 大鼠來得高，這部分可能與 SHR 年齡較大及暴露微粒後會加重肺部發炎表現有關。SHR 實驗時週齡較大為 23～24 週，SD 大鼠則較年輕為 9～10 週，在 SHR 肺部出血血管變化研究中發現，SHR 肺泡出血的情形與年紀有很大的相關性，年長的 SHR 肺泡灌洗液中紅血球及蛋白質比年輕的顯著增加（Yoshioka et al., 1989）；有研究指出，12～13 週大的 SHR 及 WKY 大鼠以吸入的方式暴露 ROFA（residual oil fly ash），即使是在沒有暴露 ROFA 的控制組與 WKY 比較，SHR 的肺泡灌洗液中有較嚴重的出血情形（Kodavanti et al., 2000）；為了加以確認是否因氣管灌注造成出血，取未經過氣管灌注 24 週大的 SHR 麻醉後進行肺泡灌洗，發現肺泡灌洗液中仍有出血情形。在實驗動物體重差異可能的影響方面，除了 9 隻國科會 SD 大鼠體重平均為 418 體重較高以外，其它實驗動物體重相差不大，20 隻樂斯科 SD 大鼠體重平均為 319，26 隻 SHR 體重介於 320～360 之間，體重的差異可能對實驗造成影響，未來進行動物實驗應盡可能採用相同動物來源之實驗動物。另外，本研究扣除出血嚴重的實驗動物，每組實驗動物數目不多，有可能因為每隻實驗動物本身的差異性影響實驗結果。

LDH 廣泛存在身體各器官組織，細胞受傷害或死亡都會釋放出 LDH，但紅血球中的 LDH 為血清中 400～100 倍，所以實驗中肺泡灌洗的部分，即使只有少量出血，若出血的紅血球有溶血現象，就會嚴重影響 LDH 偵測值，而研究中 SHR 暴露微粒後肺泡灌洗液中 LDH 顯著低於 SD 大鼠，推測與用來檢測 LDH 這部分的 SHR 檢體冰凍保存過久，部分 LDH 已衰減有關。

DNA 氧化傷害部分，SHR 之 DNA SSB 及血清 8-OHdG 都有隨總表面積增加的趨勢，但 SD 大鼠則無，且 SHR 血清 8-OHdG 不但基本值就比 SD 大鼠高，暴露微粒後造成的 DNA 損傷也都較 SD 大鼠明顯，這部分跟發炎反應一樣，可能與 SHR 年齡較大且本身抗氧化能力較低有關。Loft 的研究中認為物種、年齡、性別都會影響尿液中 8-OHdG 含量，且尿液中 8-OHdG 含量與各生物的基礎代謝率有關（Loft et al., 1993）；其它研究指出 SHR 會產生較高的氧化壓力與增加 NADPH 氧化酵素（oxidase）活性的表現，促使 O2·-在血管產生、破壞一氧化氮合成酵素及受到第一型血管收縮素 II 接受器（angiotensin II type 1 receptor）調節，這些都與增加氧化壓力有關（Touyz et al., 2004）。兩個評估 DNA 損傷的指標中，血清 8-OHdG 比 DNA SSB 在 SHR 及 SD 大鼠造成的差異性較為明顯，可能是因為 DNA SSB 在暴露後初期會較為明顯，而實驗中量測的時間是在暴露後 24 小時，可能有些 DNA 修補機制發生，而 8-OHdG 是酵素修補後切下來的產物，此時量測血清 8-OHdG 可能會比 DNA SSB 更加有顯著差異。Li 研究中發現大鼠暴露 125 μg 的 ufCB，肺泡灌洗液中的 GSH 在氣管灌注 6 小時後下降，但一天後就上升顯著高過控制組並持續維持 7 天，而肺部組織的 GSH（Glutathione）一天後仍比 6 小時更低，暴露時間的長短跟量測指標也是重要的影響因素（Li et al., 1999），故選擇不同的生物指標還必須要考慮到量測的時間點；微粒暴露造成氧化傷害這部分，在本研究中並沒有進行暴露時間的評估，所以未來的研究可考慮在不同暴露時間點量測 8-OHdG 及 DNA SSB 的變化，找出最適合的暴露時間。

總結，在非細胞系統實驗結果，各種粒徑之 ufCB ROS 的產生隨重量濃度增加而增加，在相同重量濃度下粒徑越小 ROS 的產生越高，且總表面積與 ROS 產生高度相關。動物實驗部分發現，SHR 暴露 ufCB 會加重肺部發炎及氧化傷害的表現，而臨床研究證實人類患有高血壓也會增加體內 ROS 的產生（Touyz et al., 2004），這部分應進一步探討奈米微粒對高血壓患者可能產生的健康危害。此研究結果證明微粒總表面積與肺部發炎及 DNA 損傷有相關性，尤其在 SHR 易感性實驗動物身上更為明顯，SHR 在奈米微粒暴露及氧化壓力相關研究上，應是相當適合的易感性動物模式。

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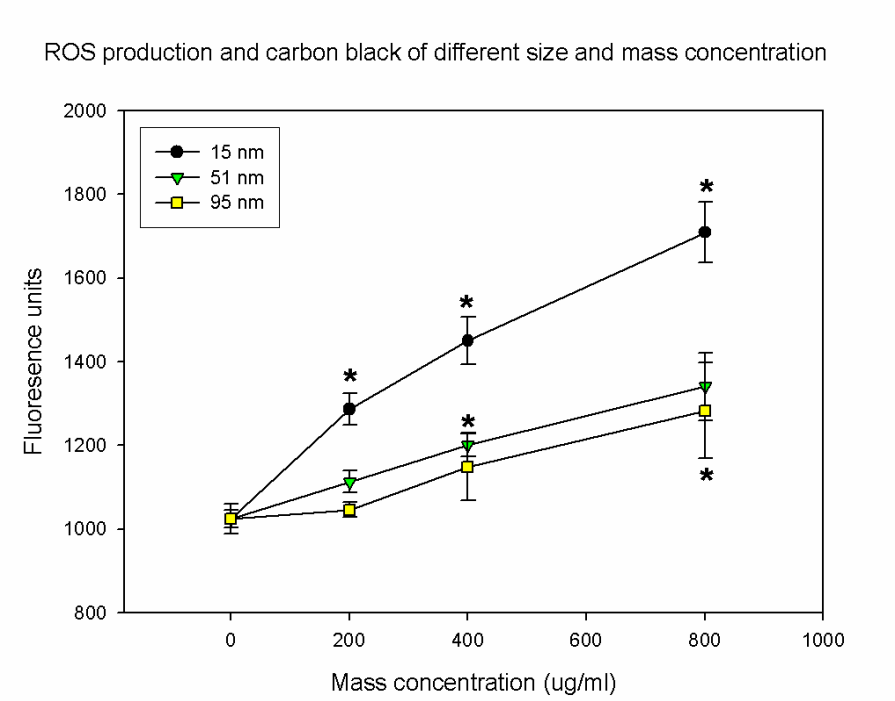
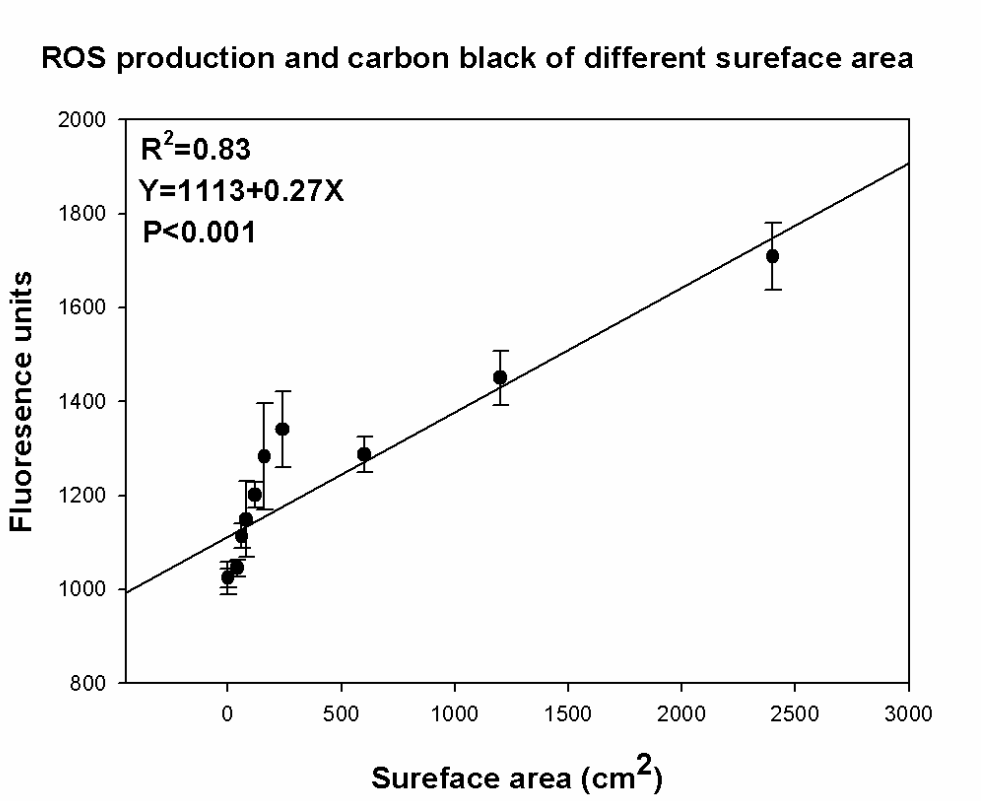
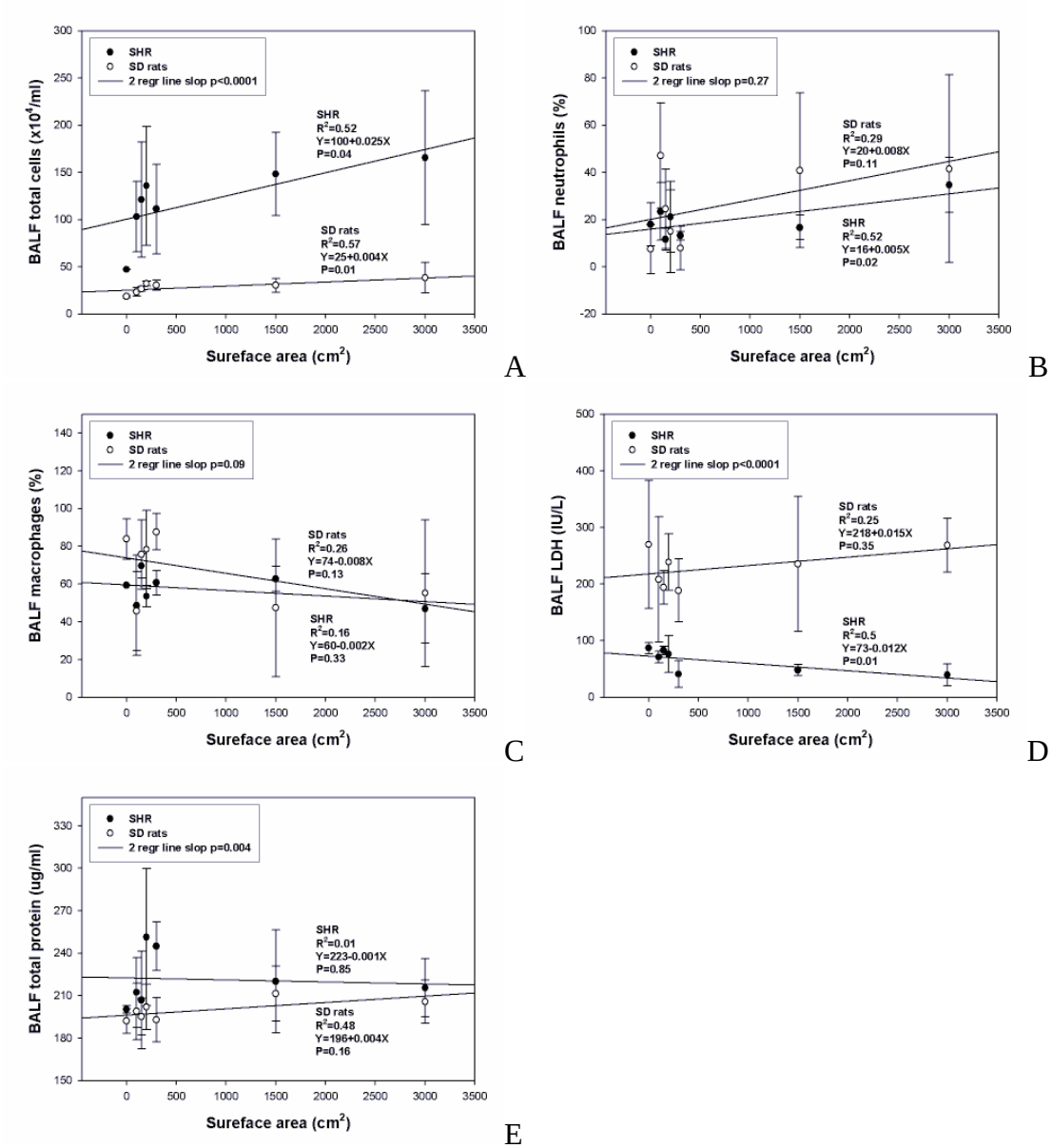


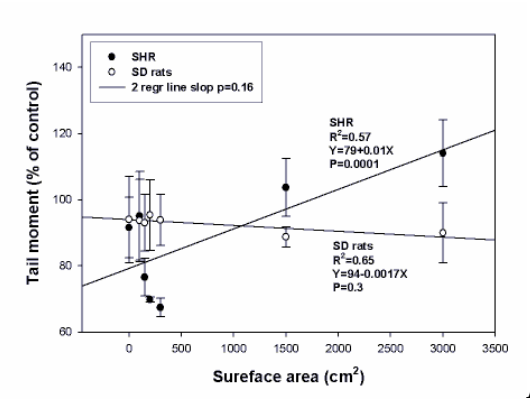
圖一、非細胞系統中暴露不同粒徑及重量濃度 ufCB 1 小時後之螢光值，以 PBS 為控制組（0μg/ml），每次每組樣本三重複，實驗數據以平均值加減標準差（mean±SD）表示，以 Student's t-tes 分析，* 表示與控制組比較 $P < 0.05$。



圖二、非細胞系統中暴露不同粒徑及重量濃度 ufCB 1 小時後，總表面積與螢光值分析結果，以 PBS 為控制組（0cm^2），每組實驗重複三次，實驗數據以平均值加減標準差（mean±SD）表示，R 為相關係數。



圖三、比較 SHR 及 SD 大鼠暴露不同粒徑及重量濃度 ufCB 24 小時後，總表面積與肺泡灌洗液中各發炎指標分析結果，以 PBS 為控制組（0cm^2），每組實驗動物數目 2～5 隻，實驗數據以平均值加減標準差（mean±SD）表示，並分析迴歸顯著性，P<0.05 表示有顯著差異。



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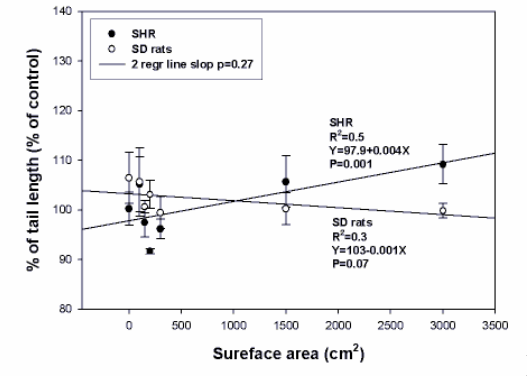

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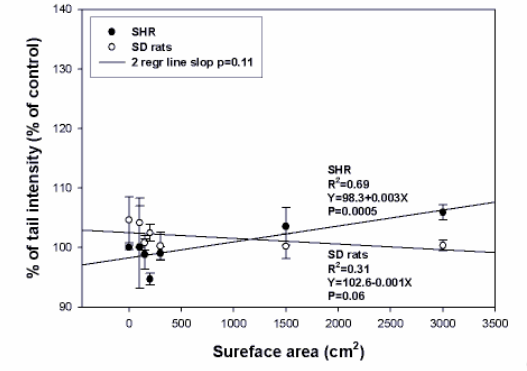

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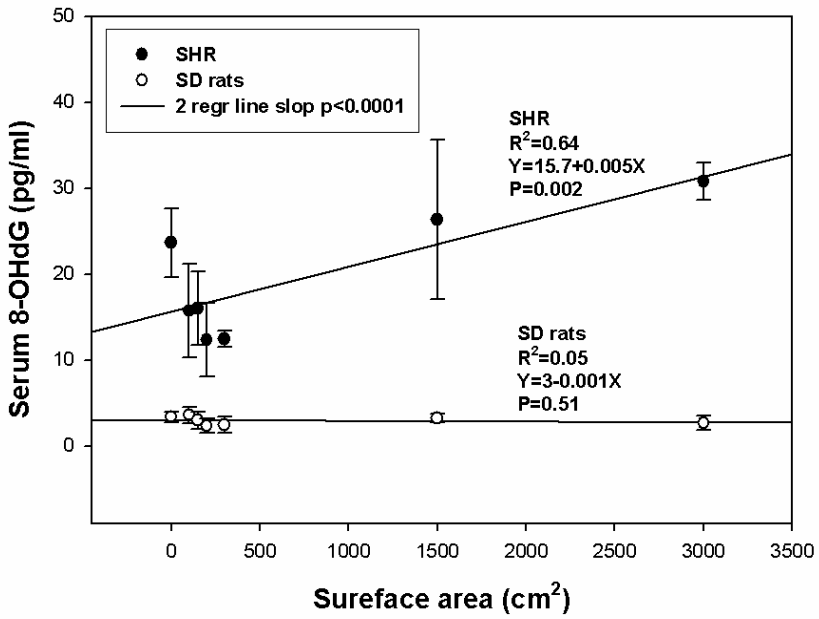
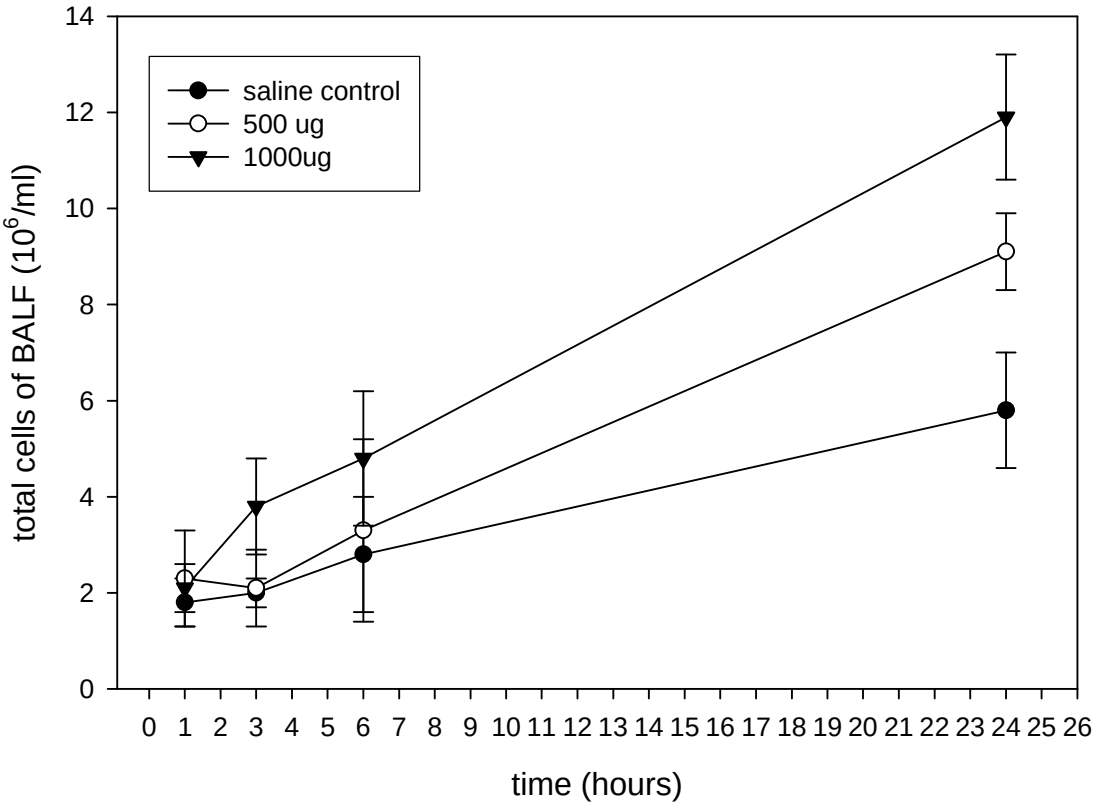
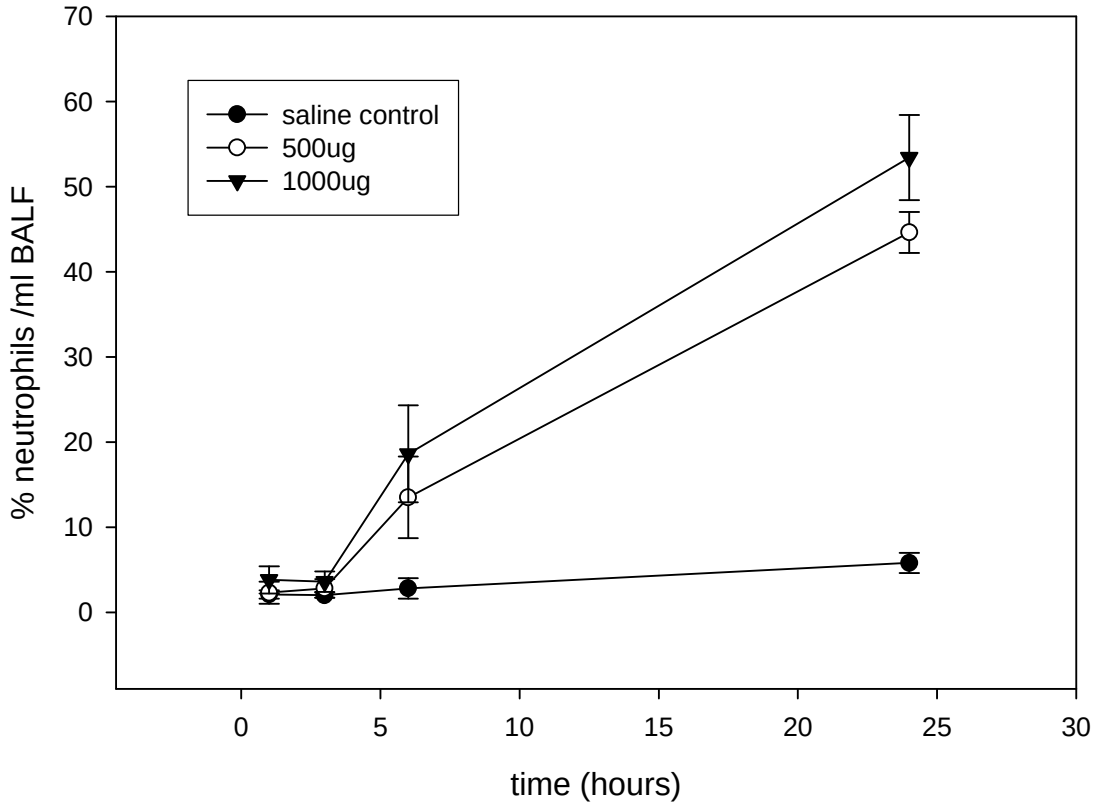
圖四、比較 SHR 及 SD 大鼠暴露不同粒徑及重量濃度 ufCB 24 小時後，總表面積與 DNA 單股斷裂各參數指標分析結果，以 PBS 為控制組（0cm^2），每組實驗動物數目 3～5 隻，實驗數據以平均值加減標準差（mean±SD）表示，並分析迴歸顯著性，P<0.05 表示有顯著差異。



圖五、比較 SHR 及 SD 大鼠暴露不同粒徑及重量濃度 ufCB 24 小時後，總表面積與血清 8-OHdG 分析結果，以 PBS 為控制組（0cm2），每組實驗動物數目 2～5 隻，實驗數據以平均值加減標準差（mean±SD）表示，並分析迴歸顯著性，P<0.05 表示有顯著差異。



圖六、氣管灌注 15nm ufCB 後，於 1、3、6 及 24 小時後犧牲，肺泡灌洗液中總細胞數與時間之關係。



圖七、氣管灌注 15nm ufCB 後，於 1、3、6 及 24 小時後犧牲，肺泡灌洗液中嗜中性球比例與時間之關係。

計畫成果自評

本計畫探討奈米微粒於實驗動物及體外細胞之毒性效應，研究成果豐碩，近三年於國內外學術研討會皆有論文發表，已達成計畫目的。然目前奈米微粒產生系統仍存在技術上之困難，無法以粉體壓縮直接產生奈米等級之微粒，未來需要針對奈米微粒產生系統進行突破，方能進一步探討奈米微粒呼吸暴露之急、慢性健康效應，以建立完整之健康風險評估資料。