行政院國家科學委員會專題研究計畫　成果報告

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 100ì g/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 100ì g/ml 及 50ì g/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 100ì g/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：

研究顯示，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，建議針對其他材質的奈米微粒毒性，以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

英文摘要

目錄

報告內容

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 2.5ì m ($PM_{2.5}$) have greater adverse health outcomes as compared to particles with a diameter below 10ì m (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

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 $\pm$ 1°C and 55 $\pm$ 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 ì g/ml in idd H_2O. Estimated surface area of instilled dose was in Table 1. Animals were anesthetized and 100 and 50ì g/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-á 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 175ì M. 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 ì g/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 100ì g/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 50ì g/ml. Similar results were observed in bronchoalveolar laveage IL-6 protein and TNF-á (Fig 3).

Fig 4 showed that a significant depletion of total GSH in lung tissue in rats with 100ì g/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 50ì g/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.

Discussion

The results showed that instillation with 100 and 50ì g/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-á 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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計畫成果自評

	Dose (μ g/ml) Surface area($cm^{2)}$	
Diameter (nm)	100 μ g	50 μ g
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.

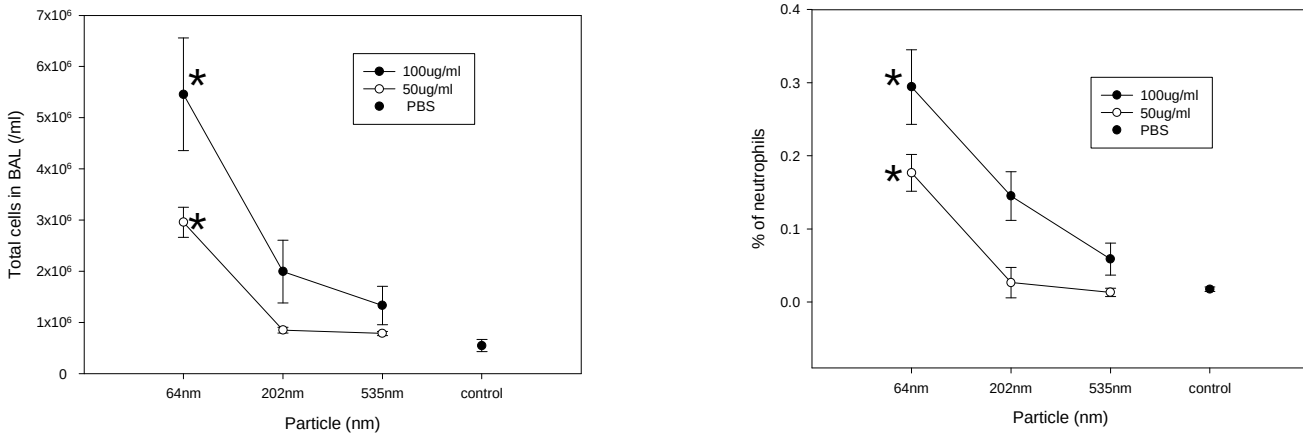


Fig 1

Total cells and percentage of neutrophils in bronchoalveolar lavage in pulmonary hypertensive rats after instillation of 100 and 50 ì g/ml of polystyrene particles.

(* $p < 0.05$)

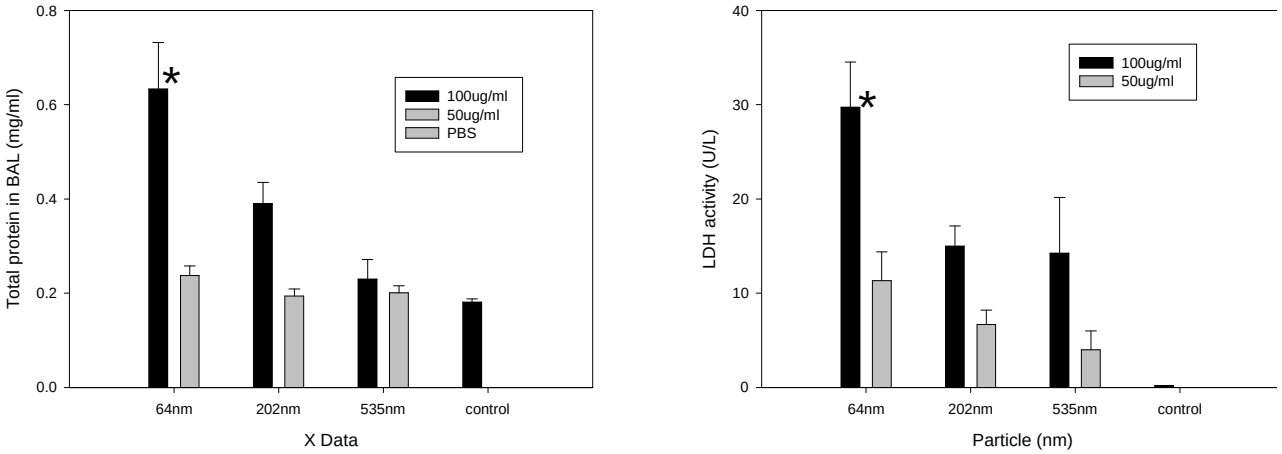
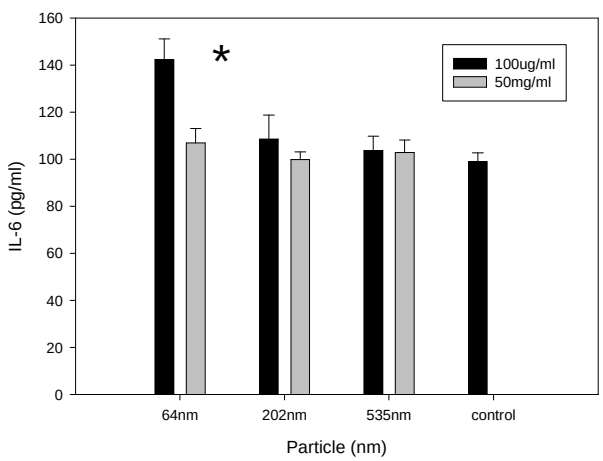


Fig 2

Total protein and LDH activity in bronchoalveolar lavage in pulmonary hypertensive rats after instillation of 100 and 50 ì g/ml of polystyrene particles.

(* $p < 0.05$)



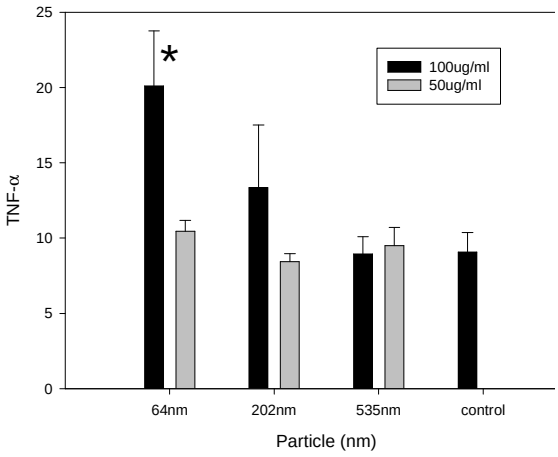


Fig 3

Proinflammatory cytokine IL-6 and TNF-á in bronchoalveolar lavage in pulmonary hypertensive rats after instillation of 100 and 50 ì g/ml of polystyrene particles .

(* $p < 0.05$)

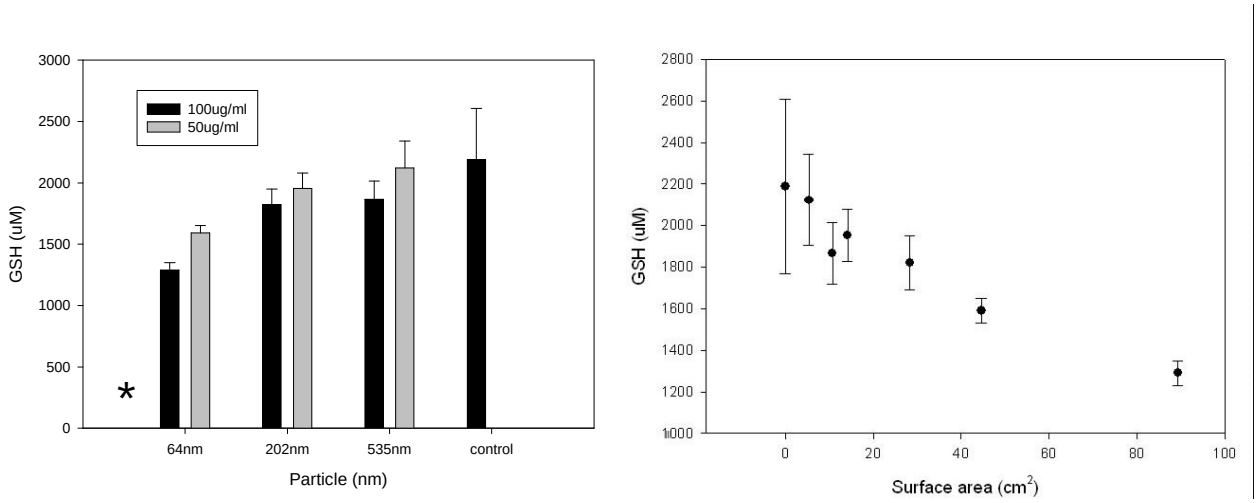


Fig 4

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

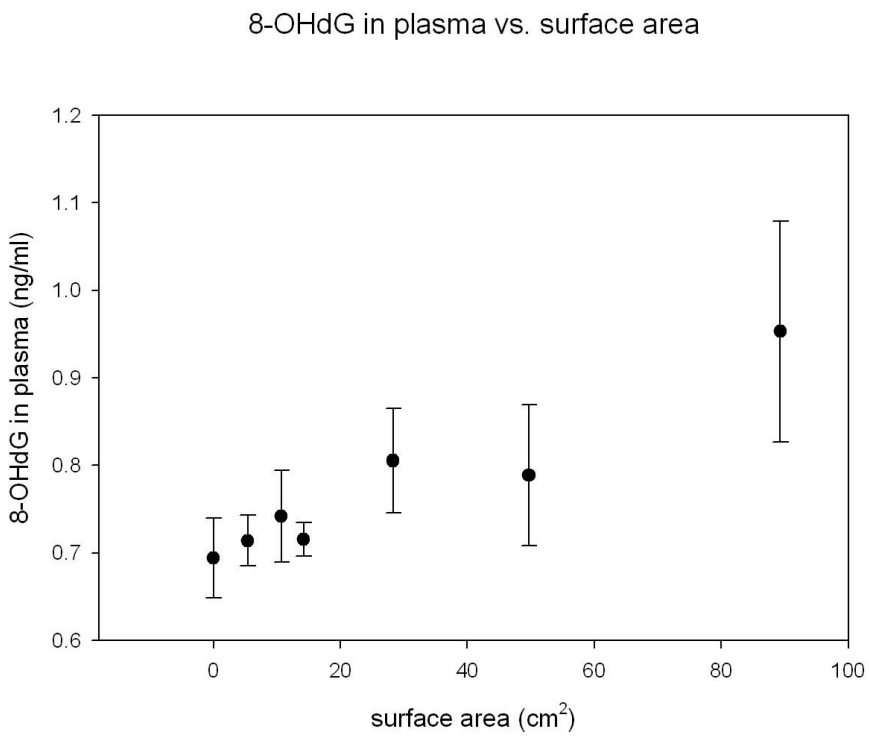


Fig 5 Mean plasma 8-OHdG response to polystyrene particles, plotted against instilled surface area dose.