# MORPHOLOGIC CHANGES IN THE LUNGS OF RATS EXPOSED TO COAL DUST: A COMPARISON OF INTRATRACHEAL INSTILLATION AND INTRATRACHEAL INSUFFLATION EXPOSURES

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## ABSTRACT

This study aims to compare two methods for exposing the lung to respirable coal dust (CD): intratracheal instillation (IT) in aqueous suspension and intratracheal insufflation (IS) of CD in air. Endpoints were histopathological changes and bronchial cellular expression of cytochrome P4501A1 (CYP1A1) in lungs exposed simultaneously to CD and a CYP1A1 inducer. For this purpose, male Sprague Dawley rats (~ 240-270 g) were exposed to 2.5 mg or 10 mg CD either by IT or IS (n=8 per group). For IT exposure, the CD dose was suspended in 0.3 ml saline and instilled into the distal trachea as an aqueous suspension using a gavage needle; whereas the IS procedure aerosolized dry CD within the trachea using a commercial device. For control, rats were exposed to saline. To induce CYP1A1, beta-naphthoflavone (BNF; 50 mg/kg) was injected intraperitoneally eleven days after CD exposures. Fourteen days after CD exposure, histopathological changes in the lung including alveolar type II (AT-II) cell hypertrophy, AT-II cell hyperplasia, and alveolitis were observed. Sections of left lungs were

stained for CYP1A1 and cytokeratin 8, a cytoskeletal protein used as a marker for alveolar type II (AT-II) cells. Terminal bronchial (Clara) cells expressing CYP1A1 and the number of AT-II cells were quantified by morphometric analysis. Histopathological changes, the number of terminal bronchial cells expressing CYP1A1 and the area of AT-II cells were unaffected by the method of exposure. These findings suggest that the pulmonary exposures by IT and IS are comparable methods for bolus delivery of respirable particles to the lungs of rats.

## INTRODUCTION

Lung is one of the vital organs that is commonly exposed to environmental toxicants particularly via inhalation (Pairon *et al*, 1994). Assessing the different means of delivery of particles to induce pulmonary exposure is extremely important in inhalation toxicity data, particularly to study the induced CYP1A1 expression in pulmonary cells, which is recently involved in lung carcinogenesis (Bjelogrlic *et al*, 1993). CYP1A1 is an enzyme that is induced by polycyclic aromatic hydrocarbons, such as those in cigarette smoke into a highly reactive metabolite (liang *et al*, 2003). This reactive intermediate compounds can react covalently with DNA nucleotides producing mutations and cancer (Wang *et al*, 1995). We have recently demonstrate that exposure of rat lung to CD by IT instillation suppresses the CYP1A1 expression and its dependent activity (7-ethoxyresorufin – *O*- deethylase) (Ghanem *et al*, 2004). Intratracheal instillation is most likely to deliver an exact dose of a compound to the lungs of an experimental animal (Sabaitis *et al*, 1999). Two well known pulmonary exposures are nowadays used for

inhalation toxicity investigations, the intratracheal instillation (IT) and intratracheal insufflation (IS). Instillation procedure is limited by the fluid volume that can be given safely, and instilled liquids distribute according to gravity. In contrast, aerosolization (insufflation) procedure can deliver larger volumes over longer periods and aerosols distribute according to ventilation (MacIntyre, 2001**)**. However, the simplicity of the IT procedure and its requirement of only microliters of a compound to generate a meaningful and reliable dose-response suggest that IT may be an alternative method for acute inhalation toxicity evaluation of materials that may present inhalation hazards from liquid or solid aerosols (Sabaitis *et al*, 1999). Because exposure by aerosolization requires longer periods to deliver certain material, the intratracheal instillation was suggested to facilitate the exposure process by shortening the time required for delivery and thereby expedite the inhalation toxicity studies. In this study, we compare the using of IT and IS for studying the morphologic changes associated with CD exposure as well as the cellular expression of CYP1A1 in lungs simultaneously exposed to CD particles and BNF (CYP1A1 inducer).

## MATERIALS AND METHODS

### *Animals*

Fourty male Sprague-Dawley rats (~220-270g) were purchased from Hilltop Labs (Scottdale, PA). The rats were housed in ventilated shoebox cages on autoclaved hardwood (Beta chip) and cellulose (Alpha-Dri) bedding in HEPA filtered, ventilated cage racks (Thoren). Food and water were supplied

*ad libitum.* Rats were allowed to acclimatize in their cages for at least 7 days before the experiment.

***Experimental design***

Rats were randomized into five groups of 8 rats each by using a research randomizer program (www.randomizer.org). For ITI, 2 groups were exposed to small (2.5mg) and large (10mg) dose of CD / rat suspended in 0.3 ml sterile saline. 2 other groups were intratracheally insufflated with 2.5 and 10 mg CD powder / rat. Eleven days later, rats were injected i.p. with the CYP1A1 inducer, beta-naphthoflavone (BNF; 50 mg/kg BW) suspended in filtered corn oil or injected with filtered corn oil. Three days after BNF injection, rats were euthanized and the right lung lobes were chopped and homogenized to collect lung microsomes, whereas the left lungs were air-way perfused with 10% neutral buffered formalin for histopathology.

***Coal Dust Particles****:*

Coal dust was obtained from the Pittsburgh coal seam. 98.9% of the particles were less than 5 microns in diameter with 17.9% of the particles less than 1 micron in diameter. The surface area was 7.4 $m^2$/g. The particles contained 0.34 % total iron of which 0.119 % was surface iron. By number percentage, 2.3% of the particles were silica. The particles were weighed, placed in a scintillation vial, covered with foil and heat-sterilized in an oven at 160 ºC for 2 hours. CD suspensions were prepared from heat-sterilized

samples using non-pyrogenic sterile saline (Abbott Laboratories, North Chicago, IL).

***Intratracheal Instillation and Insufflation***

The CD particles were suspended in sterile saline at a concentration of 8.3 and 33.3 mg/ml. Rats received either 0.3 ml of this suspension (~2.5 and 10 mg/rat) or equivalent dose of sterile saline (vehicle). The rats were anesthetized by i.p. injection of sodium methohexital (Brevital, Eli Lilly Indianapolis, IN) and were intratracheally instilled using a 20-gauge, 4-inch ball-tipped animal gavage needle as previously described (Porter *et al*, 2002). On the other hand, the IS procedure aerosolized dry powder within the trachea using a dry powder insufflator (Model DP-3, Penn-Century, Inc, Philadelphia, PA). Because coal dust is black, the distribution of coal dust to both the left and right lungs was confirmed by gross examination of exposed lungs at necropsy.

***Beta-Naphthoflavone (BNF) Preparation***

Twenty four hours before i.p. injection, solutions of 5 % (50 mg/ml) BNF (Sigma-Aldrich Co., St. Louis, MO) in corn oil were prepared. The corn oil used was filtered with non-pyrogenic Acrodisc 25 mm syringe filter (0.2 µm in diameter) (Pall Gelman Sciences, Ann Arbor, MI) to assure sterility. The solution suspension was vortexed until the particles were evenly suspended and then sonicated in Ultronics sonicator (Mahwa, NJ) for 15 minutes before injection. BNF solutions were injected once, i.p., at a dose of 50 mg/kg 3 days before sacrifice.

***Euthanasia***

Euthanasia was conducted by i.p. injection of 0.5 ml 26% sodium pentobarbital (Sleepaway®, Fort Dodge Animal Health, Fort Dodge, IA) 14 days after CD exposure.

***Necropsy***

The lungs and attached organs, including tracheobronchial lymph node, thymus, heart, aorta and esophagus, were removed. The right lung lobes were collected and weighed at necropsy for microsomal preparation, while the left lung lobe was inflated with 3 cc of 10% neutral buffered formalin (NBF). Fixed tissues were trimmed the same day, routinely processed in a tissue processor, and embedded in paraffin the following morning. 5-micrometer sections were prepared and used for immunofluorescence as previously described (Ghanem *et al*, 2004).

***Histopathology:***

Lung tissue sections of control and CD-exposed rats were routinely stained with hemotoxylin and eosin (H&E) for histopathology. The stained slides were examined and interpreted by a board-certified veterinary pathologist blinded to the exposure status of the individual slides. Changes assessed in each slide included: AT-II cell hyperplasia and hypertrophy and alveolitis (inflammation), Histopathologic changes were scored for severity and distribution from zero to five as previously described (Hubbs *et al*, 1997). Briefly, severity was scored as none (0), minimal (1), mild (2), moderate (3), marked (4), or severe (5). Distribution was scored as none (0), focal (1), locally extensive (2), multifocal (3), multifocal and coalescent (4), or severe (5). The pathology score is the sum of the severity and distribution score.

***Microsomal Preparation***

Lung microsomes were prepared for determination of total lung microsomal protein. Lung microsomes were prepared as previously described (Flowers and Miles, 1991; Ma *et al*, 2002; Ghanem *et al*, 2004). The lung microsomes were obtained by differential centrifugation to remove the cell nuclei and debris and reduce the mitochondrial contamination. Then microsomal pellets were re-suspended in the incubation medium (145 mM KCl, 1.9 mM $KH_2PO_4$, 8.1 mM $K_2HPO_4$, 30 mM Tris-HCl, and 3 mM $MgCl_2$; pH 7.4) at a ratio of 1gm lung/1ml medium and frozen at -80C until assayed.

***Determination of the Total Lung Proteins***

The bicinchoninic acid (BCA) method was used for determination of protein content of lung microsomes as previously described (Ma *et al*, 2002 and Smith *et al*, 1985) using a BCA protein assay kit (Pierce, Rockford, IL) in a spectra Max 250 spectrophotometer (Molecular Devices Corporation, Sunnyvale, California). Bovine serum albumin was used as the standard.

***Indirect Immunofluorescence Technique***

Paraffin-embedded, formalin-fixed sections from the left lung lobe were used for

immunofluorescent detection of CYP1A1 and cytokeratins 8, which is cytoskeletal protein used as a marker of alveolar type II cells (AT-II) (kasper *et al*, 1993). The procedure was conducted as previously described (Ghanem *et al*, 2004) with little modification. The primary antibodies used for CYP1A1 and cytokeratins 8 staining were a polyclonal, affinity purified highly cross-absorbed rabbit anti-rat CYP1A1 (Xenotech, Kansas City, US) and sheep anti

human cytokeratin 8 (The Binding Site, Birmingham, UK). For CYP1A1 detection, Alexa 594-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, US) was applied at dilution of 1:20 to give a red fluorescence. For cytokeratin 8 detection, Alexa 488-conjugated donkey anti sheep (Molecular Probes, Eugene, OR, US) was applied as a secondary antibody at dilution of 1:50 to bind to primary antibody to give green fluorescence.

***Morphometric analysis***

Morphometric analysis was conducted using commercial morphometry software (Metamorph Universal Image Corporation). By the aid of this program, the number of terminal non-ciliated bronchiolar (Clara) cells expressing CYP1A1 was counted per mm of the basement membrane (five images per slide were used). In addition, the number of AT-II cells (with cytokeratin 8 expression) was counted per field and five fields were counted per slide and the average was used for individual statistics. Similarly, the area of cytokeratin 8 expression in AT-II cells was quantified and expressed as square millimeter.

***Statistical Analysis***

Pairwise comparisons of each dose to the control group and a comparison between IT and IS groups were analyzed, using one-way analysis of variance as previously described (Ghanem *et al*, 2004). All differences were considered statistically significant at $p<0.05$.

## RESULTS

***1-Effect of IT and IS on Histopathological Changes***

By histopathology, the IT has no significant effect on AT-II cell hyperplasia and hypertrophy relative to IS in all exposed groups (figure 1A). However, there was a high significant increase ($p<0.001$) of AT-II cell hyperplasia and hypertrophy in groups exposed to 10 mg CD compared to their counterpart groups exposed to 2.5 mg (figure 1A), suggesting that CD exposure enhance AT-II cell hyperplasia and hypertrophy in rat lungs. Similarly, the severity and distribution of alveolitis was not affected by the method of exposure either by IT or IS (figure 1B). Alveolitis appear as alveolar histiocytosis associated accumulation with of brown pigment (CD particles) in alveolar macrophages and, less frequently, in the interstitium (figure 2). There was a significant increase ($p<0.05$) of alveolitis in groups exposed to 10 mg CD compared to their counterpart groups exposed to 2.5 mg except in the group exposed by IT to CD 10 mg/oil, there was non significant increase compared to its counterpart group (CD 2.5 mg/oil) (figure 1B)

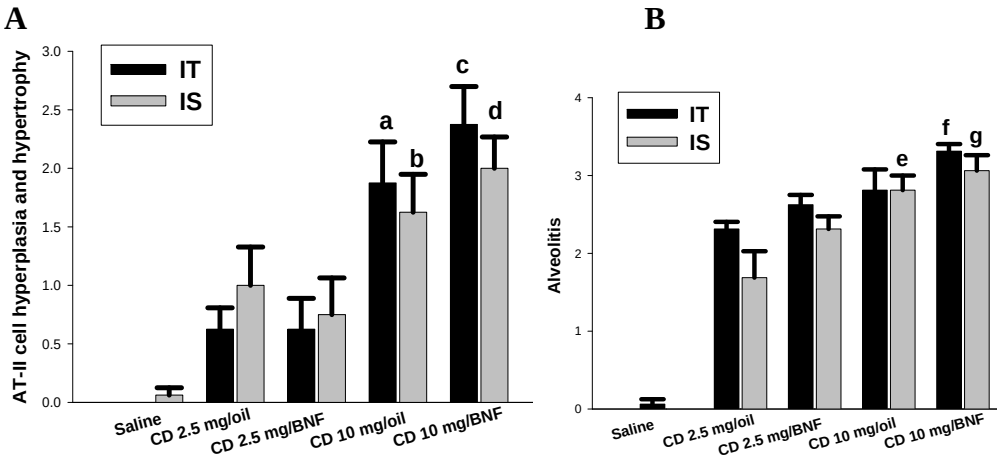


Figure1: Effect of IT and IT of CD on histopathological changes. A-The IT has no significant effect on AT-II cell hyperplasia and hypertrophy relative to IS in

all groups. Letters a, b, c and d indicate a high significant increase of AT-II cell hyperplasia and hypertrophy in rats exposed to 10 mg CD compared to their counterpart exposed to 2.5 mg. B- The IT has no significant effect on alveoltis relative to IS in all groups. Letters e, f and g indicate a significant increase of alveoltis in groups exposed to 10 mg CD compared to their counterpart groups exposed to 2.5 mg. Results are mean ± SE (n=8).

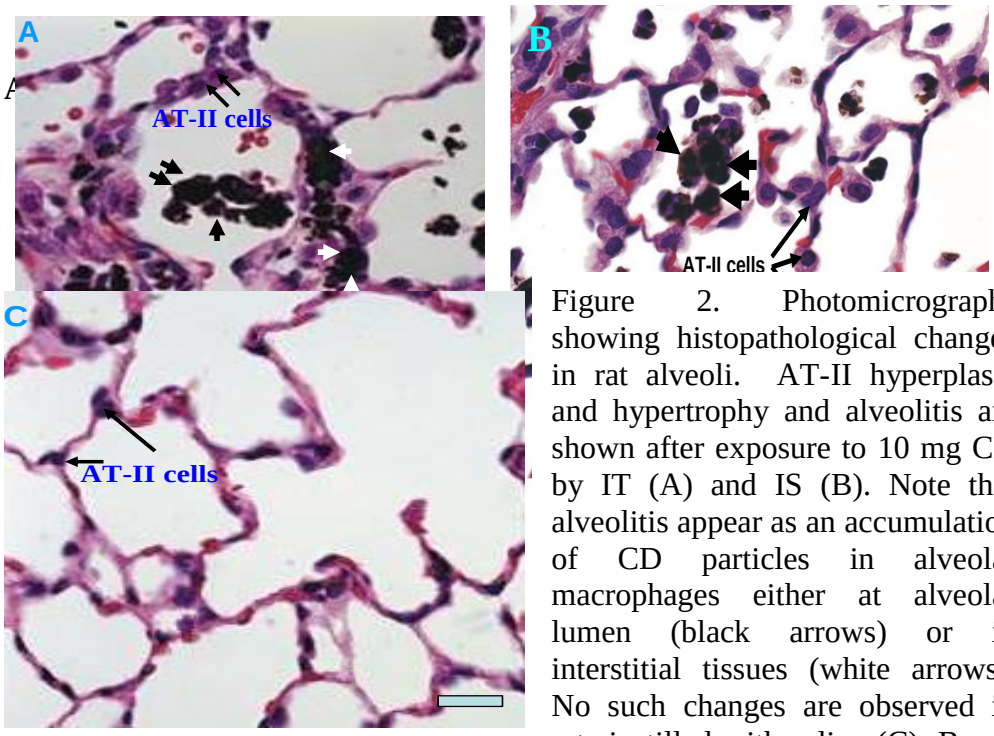


Figure 2. Photomicrographs showing histopathological changes in rat alveoli. AT-II hyperplasia and hypertrophy and alveolitis are shown after exposure to 10 mg CD by IT (A) and IS (B). Note that alveolitis appear as an accumulation of CD particles in alveolar macrophages either at alveolar lumen (black arrows) or in interstitial tissues (white arrows). No such changes are observed in rats instilled with saline (C). Bar is 20 µm.

***2-Effect of IT and IS on Terminal Bronchial (Clara) Cells Expressing CYP1A1***

No significant changes in the number of Clara cells expressing CYP1A1 were observed in groups exposed to CD by IT versus IS (figure 3). On the other hand, groups received BNF (a CYP1A1 inducer) have a

significant increase ($p<0.05$) in the number of Clara cells expressing CYP1A1 compared to their corresponding groups received oil suggesting that BNF induce CYP1A1 expression in rat terminal non-ciliated bronchiolar cells (figure 3). Examples of immunofluorescent photomicrographs of terminal bronchiolar cells (green) expressing CYP1A1 (red) are shown in figure 4 which appear in yellow color, which is a co-localization (concurrent occurrence) of green (cytokeratin 8) and red (CYP1A1) fluorescence.

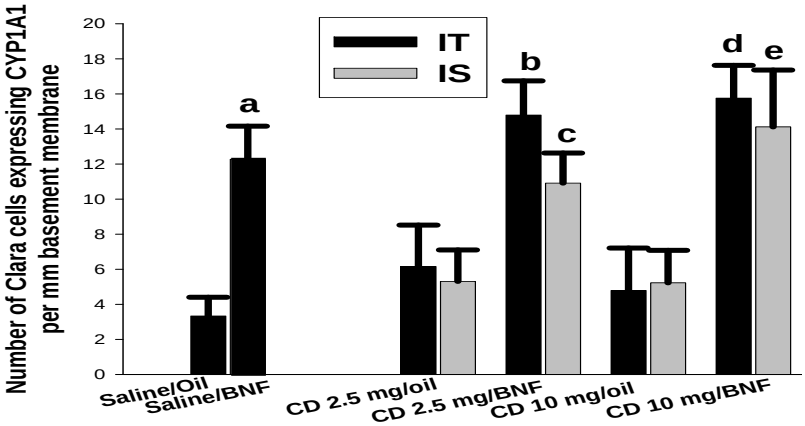


Figure 3. Effect of IT and IS on terminal non-ciliated bronchiolar (Clara) cells expressing CYP1A1. No significant change in the number of Clara cells expressing CYP1A1 in groups exposed by IT versus IS. Letters a, b, c, d and e indicate that groups received CD by either IT or IS and injected with BNF (saline/BNF, CD 2.5 mg /BNF and CD10 mg /BNF) have a significant higher ($p<0.05$) number of Clara cells than corresponding groups received oil vehicle. Results are means+ SE (n=8).

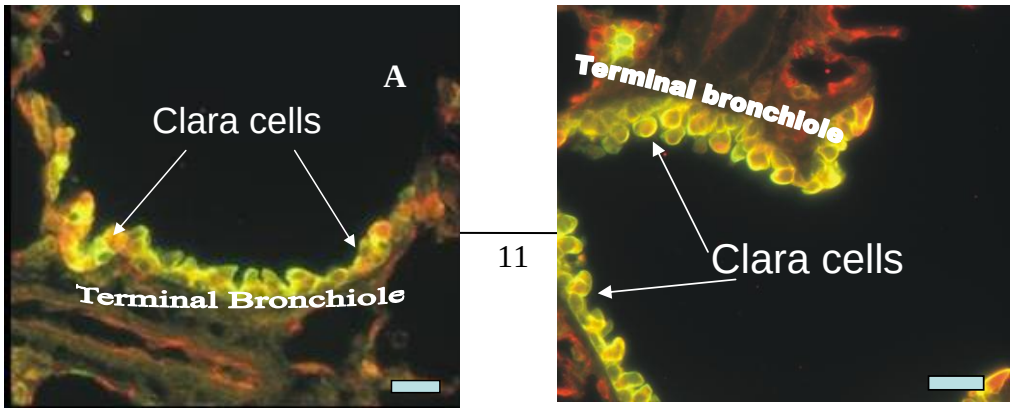

Figure 4. Representative immunofluorescent images showing the terminal non-ciliated bronchiolar cells expressing CYP1A1 in rat lungs exposed to 10 mg CD by IT (A) and IS (B). Notice that the number of cells expressing CYP1A1 (which appear yellow due to co-localization of the green and red color) is not significantly affected the method of exposure either by IT or IS. Bar is 20 µm.

***3-Effect of IT and IS on Cytokeratin 8 Expression in Rat Alveoli***

The area of cytokeratin 8 expression in rat alveolar wall (as a marker of AT-II cells) measured as square millimeter (figure 5A) and the number of AT-II cell per microscopic field (figure 5B) were not significantly affected by the method of pulmonary exposure either by IT or IS (figure 5A and 5B). However, the number of AT-II cell per microscopic field was significantly increased ($p < 0.05$) in all groups received CD particles either by IT or IS compared to control (figure 5B) suggesting that CD exposure produce AT-II cell hyperplasia in rat lungs. Examples of cytokeratin 8 expression in AT-II cells are shown in figure 6 and appear as green fluorescence.

A B

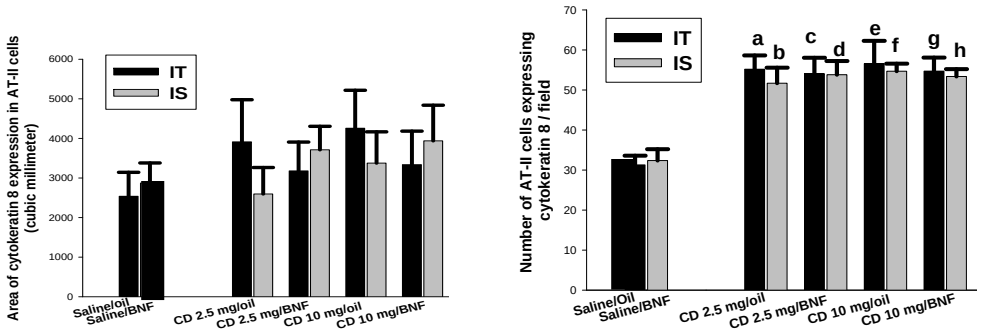


Figure 5. Effect of IT and IS on cytokeratin 8 expression quantified by morphometric analysis. No significant change in the area of cytokeratin 8 expression (A) and the number of AT-II cells (B) in groups exposed by IT versus IS. Letters a, b, c, d and e indicate that groups received CD by either IT or IS have a significant higher number ($p<0.05$) of AT-II cells than corresponding groups received saline. Results are means $\pm$ SE (n=8).

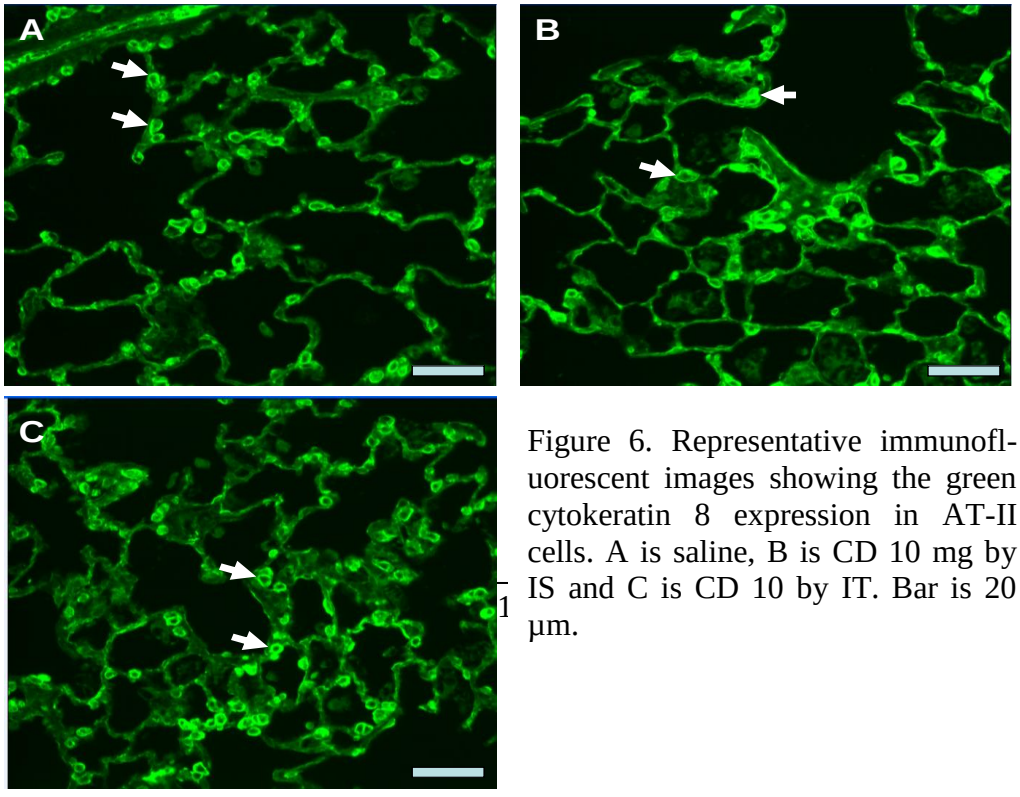


Figure 6. Representative immunofl-uorescent images showing the green cytokeratin 8 expression in AT-II cells. A is saline, B is CD 10 mg by IS and C is CD 10 by IT. Bar is 20 µm.

***Effect Of IT and IS on Total Microsomal Lung Protein***

The total lung proteins measured as mg per lung in lung microsomes was not significantly affected by the method of pulmonary exposure either by IT or IS (figure 7).

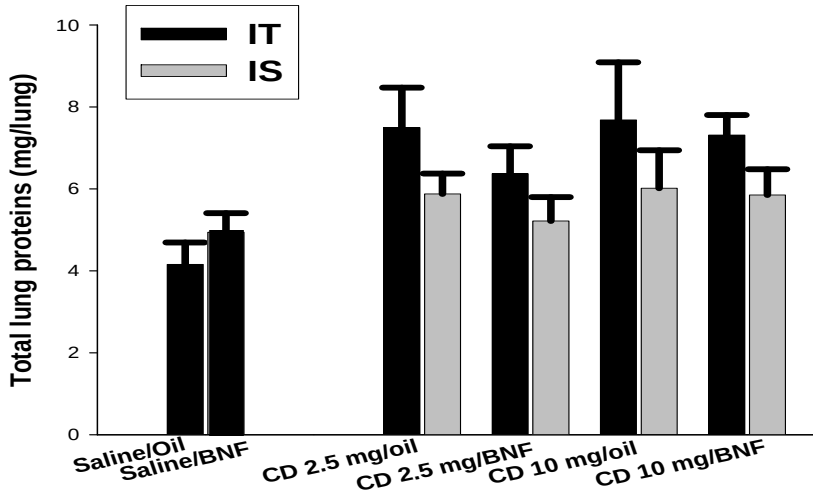


Figure 7. The total lung proteins measured as mg per lung is not significantly affected by the method of pulmonary exposure either by IT or IS. Results are mean ± SE (n=8)

## DISCUSSION

In animal studies concerning the pulmonary exposure and deposition of inhaled particles, the traditional method is to expose the animals by nose inhalation to aerosol atmosphere at closed chamber (Leong *et al*, 1997) and the

dose is calculated by the aerosol concentration by the duration of exposure (MacFarland 1976). The problem with this maneuver is that a finite dose can not be delivered. Another technique is to expose the animal by IT instillation of particle suspension, which deliver an exact dose of solution or suspension into the tracheobronchial tree (Brain and Valberg 1972). This IT instillation technique uses a 2-ml syringe with an 18-gauge oral ball-tiped feeding needle. The tip of the needle is inserted beyond the focal cords into the trachea of anesthetized rat followed by slow dripping into trachea and flowing into the lung by gravitation force (Sabaitis *et al*, 1999). By this technique, lungs produce good pulmonary responses (Taylor *et al*, 1997) and no sophisticated equipment is required (Sabaitis *et al*, 1999). Moreover, the IT instillation requires only few milligram quantity of particle suspension per animal and therefore there is no wastage of the material used (Sabaitis *et al*, 1999). However, the instilled suspension is not uniformly distributed throughout the lung tissue (Brain and Valberg 1972). A third method of pulmonary exposure has been used by Leong *et al*, 1985 to improve the distribution of inhaled aerosol by intratracheal nebulization in the lungs of dogs and monkeys, respectively. The same technique was modified and used in rats (Leong *et al*, 1998). In this study, we compare the IT instillation of particle suspension with intratracheal insufflation of dry CD powder using commercial delivery device.

Histopathological changes associated with pulmonary exposure included AT-II cell hyperplasia and hypertrophy and alveolitis (figures 1 and 2). Alveolitis is characterized by accumulation of dust-laden macrophages in the alveolar lumen and less frequently in the interstitium aggregating in clumps

of 2 or more cells (figure 2A) indicating inflammatory response. These results coincide with those obtained by Nikula *et al*, 1997. The pulmonary exposure by IT instillation or intratracheal insulation has no significant effect on the histopathological changes associated with CD exposure suggesting that the CD exposure induce similar pulmonary changes either in aerosols or in suspensions.

The terminal non-ciliated bronchiolar (Clara) cells were identified as a group of adjacent columnar non-ciliated airway epithelial cells resting on the basement membrane of the terminal bronchioles (figure 4) (Widdicombe and Pack, 1982 and Plopper, 1983). They serve as an important site for xenobiotic metabolism in the distal lung (Boyd *et al*, 1977). Moreover, these cells are the precursors of AT-II cells. For these reasons, we counted the number of CYP1A1 positive staining cells and standardized that number per micrometer of the basement membrane. The number of Clara cells expressing CYP1A1 was not significantly affected by the method of exposure, either by IT or IS (figure 3). Therefore, the presence of foreign dusts, such as respirable CD either in aerosol or aqueous suspension does not affect the number terminal bronchiolar epithelium.

AT-II cells are the most biologically important cells of lung alveoli. Its major function is to synthesize and secrete surfactant, which is a mucoid film surrounding the alveolar epithelium. This alveolar lining material (surfactant) is composed of phospholipids, proteins, and carbohydrates (Dobbs and Mason, 1979) and its major function is to reduce surface tension (Pattle, 1961). A reduction of surface tension is biologically important because it reduces the

work required for lung inflation, maintains alveolar stability and prevents alveolar edema (Clements *et al*, 1959). Moreover, following injury of alveolar type I cells, AT-II cells proliferate (Stanley *et al*, 1992) and differentiate into type I cells in order to repair the alveolar epithelium and maintain the alveolar architecture and lung function (Melloni *et al*, 1995; Thet *et al*, 1984). For these reasons, we counted the number of AT-II cells after staining their structural protein (cytokeratin 8) per microscopic field in order to assess the changes in the number associated with the CD exposure by IT or IS method (figure 5). There was no significant change in the number of AT-II cells in groups exposed to CD by IT versus IS. Moreover, the area of cytokeratin 8 expression (which is a cytoskeletal protein expressed in primitive epithelial cells and directly reflect the AT-II cell hyperplasia and hypertrophy) (figure 6) was not significantly affected. However, the number of AT-II cells was significantly higher in all groups exposed to CD either by IT or IS compared to those exposed to saline. AT-II cells generally respond to exposures of respirable particles, such as silica and coal dust, by increasing their number (hyperplasia) and size (hypertrophy) (Panos *et al*, 1990). Several mechanisms have been introduced to explain the AT-II cell hyperplasia and hypertrophy following foreign particle exposures. A possible role of the extracellular signal-regulated kinases (ERKs) was suggested by Albrecht *et al* (2001) when they found a chronic activation of phosphorylated ERKs in immunohistochemical examination of lung sections of coal dust-exposed rats. The release of alveolar macrophage-produced mitogenic factor by activated macrophages is another possible role that enhances the cell cycle proliferation and DNA synthesis in

rabbit AT-II cells (Brandes and Finkelstein, 1989). However, other factors in the bronchoalveolar lavage (BAL) of normal rats have been shown to stimulate the DNA synthesis in the primary culture of rat AT-II cells (Leslie *et al*, 1989). Panos *et al*, 1990 demonstrate a similar mitogenic effect of BAL fluid collected from normal and silica-treated rats suggesting that other factors may be involved in the silica-associated AT-II hyperplasia. The AT-II hypertrophy associated with particle exposure could be attributed to cellular enlargement during cell division (Miller *et al*, 1986), an increase in cytoplasm during preparatory stage of differentiation and transition from AT-II to type I cells, or edema and degeneration associated with cell death (Miller *et al*, 1986 and Baserga, 1985). It is well known that the cell size is increased during DNA synthesis and mitosis (Baserga, 1985) and the cell component should be duplicated during each cell cycle to maintain a uniform cell size between generations (Fraser and Nurse, 1978 and Killander and Zetterberg, 1965). Not only were the morphologic changes investigated, but the total lung microsomal proteins were also measured by spectrophotomertic assay. Again, there were no significant changes in the values of total microsomal proteins measured in lung microsomal suspensions of groups exposed to CD by either IT or IS. This result suggests that both ways of intra-pulmonary delivery have comparable changes in the total lung microsomal protein.

In conclusion, the morphologic changes in rat lungs following pulmonary exposures of CD particle and the total lung proteins were not significantly affected by the method of delivery either by IT and IS, suggesting that both methods are comparable for studying the pulmonary toxicity

associated with environmental exposure. However, the simplicity of IT technique and its relatively shorter time makes it more practical than IS procedure, which requires special equipments in addition to the difficulty of estimating the exact dose delivered to lung.

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**لملخـــص العـــــــربى**

## التغيرات المورفولوجية فى رئات الفئران المعرضة لاتربة الفحم : مقارنة بين طريقة تعرض القصبة الهوائية لمستحلب الاتربة والتعرض عن طريق التعفير

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استهدفت هذه الدراسة مقارنة بين تعرض الرئة لاتربة الفحم عن طريق تعرض القصبة الهوائية لمسحلب مائى للاتربة وعن طريق التعفير. تم تقييم التغيرات الباثولوجية الخلوية وكذلك التواجد الخلوى لانزيم السيتوكروم P450 1A1 (CYP1A1) فى الرئة المتعرضة لاتربة الفحم ومنشط لانزيم السيتوكروم CYP1A1 فى وقت واحد. ولهذا الغرض تم استخدام ذكور فئران سبراج داولى يتراوح وزنها بين 240-270 جم و تم تعرض هذه الفئران لاتربة الفحم بجرعة 2.5 و 10 مج لكل كجم من وزن الفار عن طريق حقن المستحلب وعن طريق تعفير البودرة ( 8 فئران لكل مجموعة). بالنسبة للتعرض عن طريق حقن المستحلب تم زوبان جرعة الاتربة فى 0.3 مل من محلول الملح الفسيولوجى وحقنت فى القصبه الهوائية باستخدام ابرة الحقن الخاصة. بالنسبة للتعرض عن طريق التعفير تم تعفير البودرة فى القصبة الهوائية باستخدام جهاز تعفير تجارى. فى نفس لوقت تم تعرض مجموعة من الفئران الى0.3 مل من محلول الملح الفسيولوجى فقط (كنترول). لزيادة تواجد انزيم CYP1A1 فى الخلايا تم حقن مادة البيتانافثوفلافون (بى.ان.اف) فى البروتونى بجرعة 50 مجم / كجم وذلك بعد احدى عشر يوما من التعرض للاتربة. بعد 14 يوما من التعرض للاتربة اشتملت التغيرات الباثولوجية الخلوية على زيادة فى حجم وعدد الخلايا الرئوية نوع 2 مع التهاب الحويصلات الهوائية. تم ايضا صبغ شرائح من الرئة اليسرى باستخدام تقنية الفلورسنتى المناعى للكشف عن انزيم CYP1A1 وكذلك سيتوكيراتين 8 الذى يتواجد فى الخلايا الرئوية نوع 2 ويستخدم كعلامة لتواجد هذة الخلايا. وعن طريق التحليل المورفوميترى تم عد خلايا الكلارا(التى تبطن الغشاء المخاطى للشعيبات الهوائية) التى يتواجد فيها اتزيم CYP1A1 وكذلك عدد الخلايا الرئوية نوع 2. وجد ان التغيرات الباثولوجية وعدد خلايا الكلارا التى يتواجد فيها اتزيم CYP1A1 وكذلك المساحة التى تتواحد فيها الخلايا الرئوية نوع 2 لم تتغير بطريقة التعرض سواء للمستحلب او التعفير. وبذلك تدل هذه النتائج على ان التعرض الرئوى للاتربة فى الفئران عن طريق المستحلب المائى والتعفير يعطى نتائج مماثلة من حيث التغيرات المورفولوجية الرئوية