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# Effect of Respirable Particles (Coal Dust) On Levels of Il-1β, Il-6 and TNF-α In Bronchoalveolar Lavage and Alveolar Macrophage Culture Medium of Rats

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## Abstract

Suppression of induced pulmonary cytochrome P4501A1 (CYP1A1) by exposure to coal dust (CD) and silica particles has been reported; but the exact mechanism was not fully elucidated. Previous studies demonstrated that CD exposure induced pulmonary inflammation in a dose-dependent manner. Since inflammation is associated with release of different mediators, we determined the levels of some of these inflammatory mediators in bronchoalveolar lavage fluid (BALF) of rat lungs exposed to CD. More specifically, we measured 3 cytokines, IL-1β, IL-6 and TNF-α. Therefore, male rats weighing 220-270 gm rats were intratracheally instilled with 40 mg /rat of CD particles (n = 4 per group) or vehicle (saline). Eleven days later, rats were injected intraperitoneally with the CYP1A1 inducer, beta-naphthoflavone (BNF; 50 mg/kg BW) or vehicle (oil) daily for 3 days. Rats were sacrificed 1 d after the last BNF injection and bronchoalveolar lavage was collected. The alveolar macrophages (AMs) were collected by centrifugation and were cultured in DMEM culture media. The AMs were counted by Coulter Counter multisizer II. IL-1β, IL-6 and TNF-α were quantitatively measured by using enzyme linked immuno-sorbent assay (ELISA) kits in both the BALF and in alveolar macrophage culture medium. To address the effect of CD on the nuclear protein content of AM, rats were exposed to 0, 2.5, 10, 20 and 40 mg CD/rat (n= 4 per group) and the nuclear protein was extracted from the AMs. Our results showed that the number of AMs was highly significantly increased in CD-exposed rats vs. saline-exposed rats. IL-1β was significantly increased in AM medium of rats exposed to CD plus BNF compared to those exposed to saline plus BNF. However, no significant change in TNF-α or IL-6 was detected by CD exposure. Also, the nuclear protein of AM was significantly increased in rats exposed to 20 and 40 mg/rat. These results suggest that CD exposure induces pulmonary inflammation, increases the production of IL-1β, and increase AM nuclear proteins that may be related to CD-mediated suppression of CYP1A1 induction

## Introduction

Coal is a fossil fuel mined all over the world. Coal dust (CD) generated during underground coal mining results in significant respiratory exposure of coal miners. In addition to the carbon, which is the main component, coal also contains oxygen, nitrogen, hydrogen, and trace elements, and several inorganic minerals. The trace elements may include copper, nickel, cadmium, boron, antimony, iron, lead and zinc (Sorenson *et al.,* 1999)

Suppression of the pulmonary metabolizing system of cytochrome P450 1A1 due to coal dust has been previously described (Ghanem *et al.,* 2004). However, the mechanism of suppression was not completely elucidated. Previous studies involving exposure to foreign particles showed increase in cytokines release by alveolar macrophages (AMs) and polymorphonuclear monocytes (PMN) (Vanhee *et al.*, 1995). For example, silica exposure in rats increases the production of interleukin-1beta (IL-1β) (Yucesoy *et al.*, 2002), interleukin-6 (IL-6) (Gosset *et al.*, 1991), tumor necrosis factor-alpha (TNF-α ) (Schins and Borm, 1995), and transforming growth factor-beta (TGF- β). The alveolar macrophage (AM) responds to stimuli such as coal mine dust by releasing inflammatory mediators such as cytokines, growth factors, reactive oxygen species, and eicosanoids. Some of these mediators have been associated with CYP1A1 down regulation in different cells. For example, IL-1β suppresses the induction of CYP1A1 and CYP1A2 mRNAs in isolated hepatocytes of male Sprague Dawley rats (Barker *et al.*, 1992). The reduction of CYP450 content of hepatocytes after incubation with serum of human with an acute respiratory viral infection and serum of rabbit with turpentine – induced inflammation was attributed to the presence of IL-1, IL-6 and interferon-γ (IFN -γ) (Bleau *et al*., 2000). Moreover, the TNF-α has been found to suppress CYP1A1 expression in Hepa 1c1c7 cells (Ke et al., 2001).

It has been demonstrated that the suppression of CYP1A1 induction in rat lungs is associated with pulmonary inflammation. CD increased polymorphonuclear leucocyte (PMN), alveolar macrophage (AM), Nitric oxide production, lactate dehydrogenase (LDH) and albumin in bronchoalveolar lavage fluid (BALF) (Ghanem *et al.*, 2004). Since cytokines are proinflammatory mediators, their possible role in suppression of pulmonary CYP1A1 induction caused by CD exposure is suggested.

Therefore, this study aimed to study the effect of CD exposure on rat lung through measuring different proinflammatory cytokines, more specifically IL-1β, IL-6 and TNF-α that are released in BALF and alveolar macrophage culture media of rat lungs exposed to CD.

## Material and Methods

### Animals

Male rats (~ 220-270 g BW) were kept in Animal Facility of the Collage of Vet. Med., Benha University, where food and water were supplied *ad libitum*. Adaptation period of one week was allowed before starting the experiments.

### Experimental Design

Sixteen male rats were randomized into 4 equal groups. Rats in each group were intratracheally instilled with of CD particles (CD; 40 mg/rat) (~ 16 mg / kg BW) suspended in sterile saline. Saline was instilled for control. Eleven days later, rats were injected intraperitoneally with BNF (50 mg/kg BW) suspended in filtered corn oil to induce CYP1A1. Corn oil was injected for control. Three days after BNF and corn oil injection, rats were sacrificed and bronchoalveolar lavage fluid was collected as described later. Another 20 rats were assigned into 5 groups, each of 4 rats. They were exposed to 0, 2.5, 10, 20 and 40 mg /rat CD. BALF was collected from all groups and AMs were collected by centrifugation and cultured and used to extract the nuclear proteins as described later.

### Preparation of CD Particle Suspension

Coal dust was obtained from El-Maghara mines in Aswan Governorate, Egypt. The CD particles were heat sterilized in an oven at 160 ºC for 2 h. CD suspensions were made up daily from heat-sterilized samples using nonpyrogenic sterile 0.9% saline. Suspensions were vortexed directly after preparation and shaken well before instillation.

### Intratracheal Instillation

The CD particles were suspended in sterile saline at a concentration of 133.3 mg/ml as previously described (**Ghanem *et al.*, 2004**). Rats received either 0.3 ml of this suspension (40 mg/rat) or 0.3 ml of saline (vehicle). The IT instillation was conducted as previously described **(Porter *et al*, 2002**). Briefly, rats were anesthetized by intraperitoneal injection of sodium methohexital (Brevital, Eli Lilly Indianapolis, IN)

and were intratracheally instilled using a 20-gauge, 4-inch ball-tipped animal feeding needle. Because of the black color of CD, its distribution to both left and right lung was verified at necropsy.

**Preparation of Beta-naphthoflavone (BNF)**

To prepare BNF (Sigma Adrich Co., St. Louis, MO) suspension, the vehicle (corn oil) was sterilized by filtering with non-pyrogenic Acrodisc 25 mm syringe filter (0.2 μm in diameter). Solutions of 5 % BNF in sterilized corn oil (50 mg/ml) were prepared 24 h before injection. The suspension was vortexed and then sonicated 15 minutes in Ultronics sonicator (Mahwa, NJ). Rats received BNF 3 days before killing by intraperitoneal injection.

**Necropsy of Rats**

Rats were euthanized by IP injection of 0.5 ml 26% sodium pentobarbital (~100 mg/kg, ip). The lungs and attached trachea were removed and bronchoalveolar lavage fluid was collected.

**Bronchoalveolar Lavage**

After removal of lungs and attached trachea, the BAL fluid was collected by using phosphate buffer saline as previously described **(Hubbs *et al.*, 2001**). A tracheal cannula was inserted, and BAL was performed through the cannula using ice-cold $Ca^{2+}$/$Mg^{2+}$-free phosphate-buffered saline (PBS). The first lavage was 6 ml while the subsequent lavages used 8 mL PBS until a total of 80 ml lavage fluid was collected. The samples were centrifuged (650g, 5 min at 4°C). Cells were combined, resuspended in HEPES-buffered medium (10 mM *N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid], 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, and 5.5 mM d-glucose, pH 7.4) and centrifuged a second time (650 g, 5 min, 4°C). The cells were then resuspended in HEPES-buffered medium to be ready for cell counting (**Porter *et al.*, 2002**). Cell count of alveolar macrophages was obtained using a Coulter multisizer II (Coulter Electronics, Hialeah, FL) as previously described (**Castranova *et al.*, 1990**).

**Alveolar Macrophage Culture**

Alveolar macrophage and PMN cells were collected by centrifugation of BAL fluid and then allowed to grow by incubation with 10 % fetal bovine serum (FBS)/PBS media for 2h at 37 ºC. The culture media was used for quantitative determination of cytokines; mainly TNF-α, IL-6 and IL-1β using enzyme-linked immuno-sorbent assay (ELISA).

**Determination of IL-1β, IL-6 and TNF-α by ELISA**

IL-1β, IL-6 and TNF- α were quantitatively determined by enzyme-linked immunosorbent assay (ELISA) by using ELISA kits according to the manufacture's instructions (Biosource International Inc., Camarillo, CA).. The result was read using a spectra Max 250 spectrophotometer at 450 nm wave length. Three wells per sample were loaded and the average reading was calculated for each sample and used for statistical analysis.

**Nuclear Protein Extraction of Alveolar macrophages**

Nuclear extraction of alveolar macrophages was conducted as previously described **(Amory-Rivier et al., 2000)**. Briefly, the cell suspension was centrifugate at 1500 rpm fot 5 minutes at 4 °C to collect the cell pellet after aspiration of the supernatant. The cells were then resuspended in lysis buffer for 4 minutes on ice to break the cell membranes. Centrifugation of this suspension produced the nuclear pellets at the bootom and cytoplasmic extract as the supernatant. The nuclear pellets were then resuspended in washing buffer followed by resuspension in extraction buffer containing 100 mM Phenylmethanesulfonyl fluoride (Sigma) to break up the nuclear pellet. Finally, the nuclear extract is the supernatant produced by centrifugation of the nuclear pellet suspension. This nuclear extract was deep frozen until being used for determination of total proteins as described later.

**Determination of the total proteins in nuclear extract**

The protein content of the nuclear extract was measured by the bicinchoninic acid method as previously described **(Ghanem et al., 2004)** using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA). Bovine serum albumin was used as the standard.

**Statistical Analysis**

Statistical analysis was conducted by using Sigma Stat software (SPSS Corporation, USA). One way analysis of variance (ANOVA) test was conducted to detect the significance among different groups. The difference in means was considered statistically significant when $P < 0.05$ or $P < 0.001$ **(Ghanem *et al.*, 2004).**

## RESULTS

### Alveolar Macrophages Count

The instilled rats were apparently healthy till the time of sacrifice and did not express obvious clinical signs. The number of alveolar macrophages after being pelleted by centrifugation of BAL fluid and counted by multisizer coulter was highly significantly increased in rats exposed to CD and Oil compared to those received saline and oil ($P < 0.001$). Compared to rats receiving saline and BNF, the number of alveolar macrophage was highly significantly increased in rats exposed to CD and BNF (**Figure 1**).

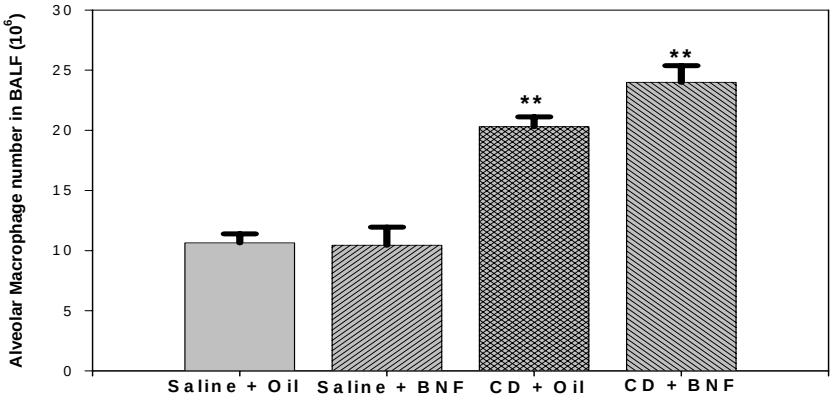


**Fig 1.** The number of alveolar macrophages is highly significantly increased in CD-instilled rats compared to saline-instilled rats. Results are means ± SE, n = 4 in all groups except in saline and oil group (n=3). ** Highly significantly different at $P < 0.001$.

### Effect of CD exposure on TNF- α, IL-1β and IL-6

The TNF- α measured by ELISA was not significantly affected by CD exposure either in BAL fluid or in AM culture media. TNF- α was significantly higher ($P < 0.05$) in AM culture media than in BALF of CD plus BNF group and non-significantly higher in the other groups (**Fig. 2A**). The IL-1β measured in AM culture media was significantly increased ($P > 0.05$) in rats exposed to CD plus BNF compared to rats exposed to saline plus BNF. Also, the IL-1â was significantly increased ($P > 0.05$) in AM culture media compared BALF in rats exposed to CD plus BNF (**Fig. 2B**).

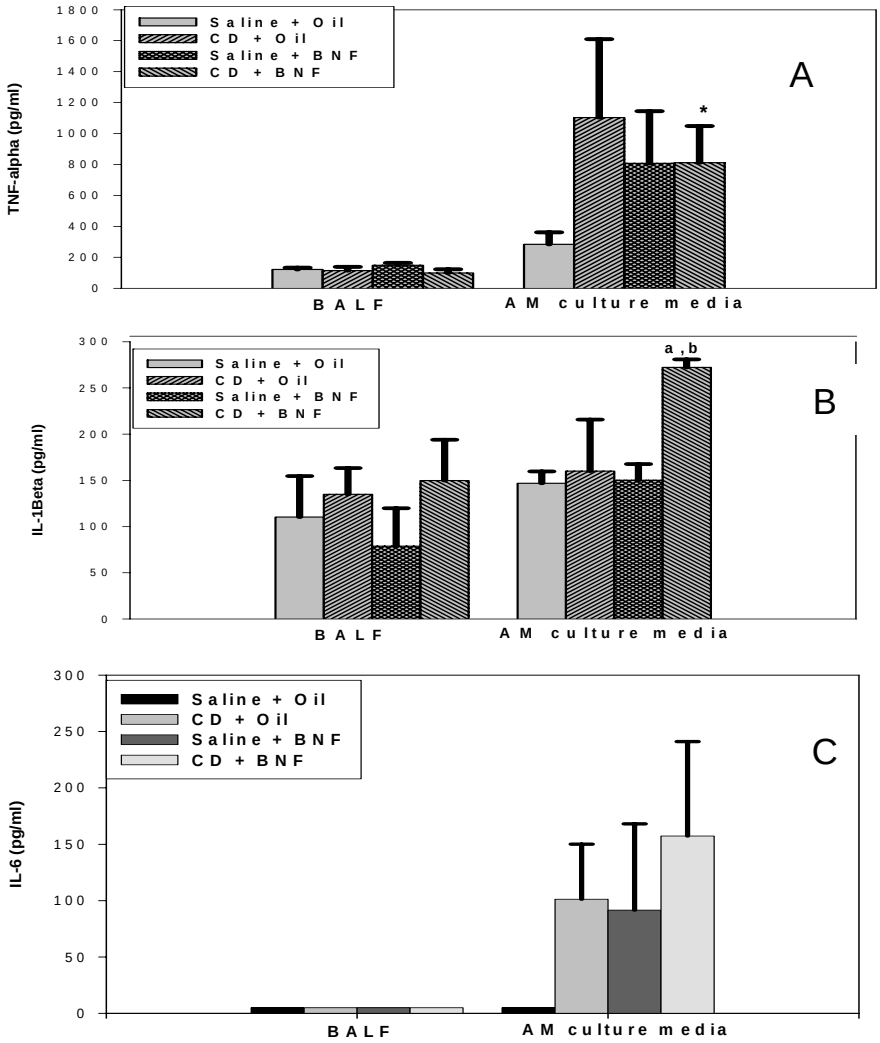


**Fig. 2.** Levels of TNF-α, IL-1β and IL-6 in BALF and AM culture media measured by ELISA.

**(A)** The TNF-α is not significantly changed by CD exposure in BAL fluid or in AM culture media but was significantly higher in AM culture media than in BALF of CD plus BNF group and non-significantly higher in the other groups. * indicates significant difference at $P < 0.05$.
**(B)** The IL-1β in AM culture media is significantly increased in rats exposed to CD plus BNF compared to rats exposed to saline plus BNF (letter a above the bar). IL-1β is significantly increased in AM culture media compared BALF in rats exposed to CD plus BNF (letter b above the bar). Letters a and b indicate significant difference at $P < 0.05$. **(C)** IL-6 is non-significantly increased in AM culture media of rats receiving CD plus BNF compared to those receiving CD plus oil and saline plus BNF. Results are represented by means ± SE, n = 4 in all groups except in saline and oil group (n=3).

The IL-6 was not detected at the BALF in all groups. In AM culture media, the IL-6 was not also detected in rats exposed to saline plus oil. However, it was non-significantly increased in AM culture media of rats receiving CD plus BNF compared to those receiving CD plus oil and saline plus BNF (Figure 2C).

**Effect of CD exposure on Nuclear Protein**

The nuclear proteins were measured in the nuclear extract of AM collected during BAL. There was a significant increase in CD-exposed rats which is significant at doses of 20 and 40 mg/rat **(Fig. 3)**.

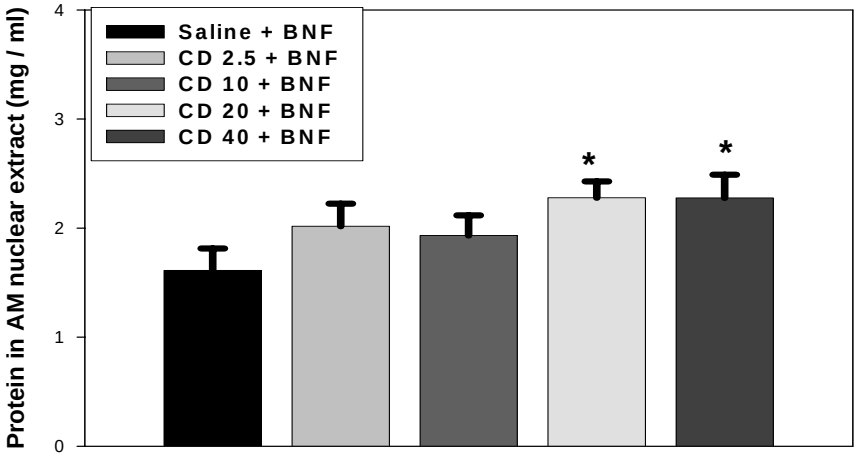


**Fig. 3.** N**uclear proteins measured in the nuclear extract of AM collected during BAL. There is a significant increase in CD-exposed rats at doses of 20 and 40 mg/rat. Results shown are means ± SE (n=4 per group). * Significantly different from saline group at P < 0.05.**

## Discussion

The breathing of coal and rock dust causes black lung, the common name given to the lung diseases pneumoconiosis and silicosis. An excruciatingly painful and deadly disease, black lung killed more than 55,000 miners between 1968 and 1990 and more than 1,000 miners still die each year (Paul, 2003). In BALF and AM culture medium, three important cytokines were quantitatively measured by ELISA kits. These included IL-1β, IL-6 and TNF-α .Selecting these three mediators was based upon their potent depressing effect on CYP enzymes in adult human hepatocytes (Abdel-Razzak *et al.*, 1993).

The number of alveolar macrophages is highly significantly increased in CD-instilled rats compared to saline-instilled rats**.** Moreover, the recovery of AM from miner's lungs by BAL was significantly greater than that from control subjects. The results of these studies suggest that occupational inhalation of Coal dust may increase total lung eicosanoid and cytokine levels and reduce the reactivity of AM to bacterial endotoxin. Furthermore, coal dust induces changes in both eicosanoid and cytokine release may be subject to pharmacological modulation **(Kuhn *et al.*,** 1995). Similarly, Schins and Borm (1999) mentioned that CD particles are also known to stimulate the macrophage production of various factors with potential capacity to modulate lung cells and/ or extracellular matrix and a number of factors that have been shown to stimulate and/ or inhibit fibroblast growth or collagen production such as TNF.

On the other side, Rao *et al.* (2005) detected that, there was no significant increase in the number of alveolar Ams in BAL fluid under any conditions of diesel exhaust particles (DEP) exposure. Chronic and acute exposure to coal mine dust by workers can precipitate the release of inflammatory mediators from pulmonary white blood cells. The alveolar macrophage (AM) is a pivotal pulmonary defense cell in this regard that responds to the inhalation of dust particles through phagocytosis of the dust particle and the release of a number of chemical mediators that help counteract the harmful effects of inhaled foreign particles. The AM can synthesize and release a host of proinflammatory and antiinflammatory factors when activated by external stimuli. These include cytokines, growth factors, and products of arachidonic acid metabolism (Sybille *et al.*, 1990). These factors inherently send signals to other white cells through an autocrine and paracrine route to participate in the neutralization of the dust particles' effects on the lung. Their previous studies *in vitro* with human cells obtained by bronchoalveolar lavage showed that both the eicosanoids and cytokines are released in substantial amounts from AM when exposed to mineral dust such as silica. It is apparent from the calcination studies that the physical properties of dust can also play an important direct role in this activation process. The alveolar macrophage (AM) responds to stimuli such as coal mine dust by releasing inflammatory mediators such as cytokines, growth factors, reactive oxygen species, and eicosanoids. AM produces primarily prostaglandin $E_2$, thromboxane $A_2$, and leukotriene $B_4$ as part of the cellular response to an inflammatory stimulus (Laurence *et al.*, 1994).

Of all cytokines analyzed, the IL-1β was the only one that was significantly increased in AM culture media of rats receiving CD plus BNF compared to rats receiving saline plus BNF. This result suggests a potent relationship between IL-1β and the CYP1A1 downregulation. The suppression of CYP1A1 induction by IL-1β has been frequently demonstrated both *in vitro*. IL-1β strongly inhibited the basal 7-ethoxyresolrufin - *O* - deethylase activity (CYP1A1-dependent metabolic activity) and fully blocked its induction by phentobarbital in primary culture of adult rat hepatocytes (Abdel-razzak et al., 1995). IL-1β, in addition to other cytokines, antagonized the polycyclic aromatic hydrocarbon-mediated induction of CYP1A gene expression in human hepatocytes (Abdel-razzak et al., 1994). Neutralization experiment showed that IL-1β together with IL-6 in rabbit serum with turpentine-induced inflammation contributed to the decrease in CYP450 content of rabbit hepatocytes (Bleau et al., 2000). Moreover, IL-1β was the most potent cytokine in regard to CYP1A1/2 mRNA suppression through a transcriptional repression mechanism in cultured rabbit hepatocytes (Calleja et al., 1997). The mechanism by which IL-1β suppresses CYP1A1 induction is not fully investigated. It was proposed that cytokines play an important role in transcriptional regulation of CYP1A1 induction. Reduction of transcriptional rate of CYP1A1 expression by IL-1β has been previously described (Barker et al., 1992). IL-1β can possibly inhibit the functional activity of CYP450s as well as increasing the heme moiety degradation by inducing heme oxygenase (Rizzardini et al., 1993).

On the contrary of our results Takano et al. (2002) found no significant change in IL-1β, ICAM-1, and MIP-1mRNA levels in the lung tissue at 24 hr. We measured the expression of four other genes) *IL-6*, *GM-CSF*, *ICAM-1*, and *RANTES*) in lung tissue after lavage and found no change in the mRNA levels of these genes under any of the exposure conditions. Similarly, there was no increase in mRNA levels of *TNF*- in BAL cells. Our observations concerning TNF- RNA level expression are consistent with observations that there is no increase in TNF -at the protein level in AMs after *in vivo* (Yang et al. 2001) or *in vitro* DEP exposure (Yang et al. 1997). In another study, cytokine levels were determined in AMs and lung tissue after inhalation exposure of DEPs for 1 month and 3 months in mice (Hiramatsu et al. 2003), which showed minimal changes in *TNF-* , *IL-1*β, and *IL-10* expression.

The IL-6 was not detected at the BALF in all groups. In AM culture media, the IL-6 was not also detected in rats exposed to saline plus oil. However, it was non-significantly increased in AM culture media of rats receiving CD plus BNF compared to those receiving CD plus oil and saline plus BNF (Figure 2C). Huang and Zhang (2003) indicated that coal dust can stimulate IL-6 release from mouse epidermal JB6 cells and human lung epithelial A549 cells, and the coal-induced IL-6 increase may involve ERKs and p38 MAPK pathways. Similarly, Gurel et al. (2004) showed that coal dust increased fibroblast proliferation, extracellular matrix synthesis and secretion of proinflammatory factors. So the effect of coal dust is not restricted to the lungs, but it also affects various systems. Especially its YL 6 and TNF-a increasing effects make them think that CD may cause inflammation and alterations in hemodynamic system.

Human studies suggest that coal dust contains stable radicals and is able to induce reactive oxygen species that may cause DNA damage. Coal mine dust can cause cytotoxicity and induce the release of mediators from inflammatory cells; however, these effects are not predictable from its quartz content alone. *In vitro*, the cytotoxicity of quartz is clearly inhibited by the presence of coal dust, while the inflammatory activity is dependent on yet unidentified parameters. The release of cytokines and growth factors most probably contributes to pneumoconiosis development. Reactive oxygen species also can inactivate α-1-antitrypsin and bronchoalveolar leukocytes from rats inhaling coal mine dust had increased secretion of connective tissue proteases, leading to the development of emphysema. (IARC, 1997)

On the other hand, Vanhee *et al.* (1995**)** found that the positive cells of INF and IL-6 found in BALF was significantly higher for patients with progressive massive fibrosis (PMF) than for those with SP of normal controls, and was correlated with cytokine concentrations in supernatants from alveolar macrophages (AM). These data confirm that TNF and IL-6 production is increased in the lungs of Pneumoconiotic patients. Moreover TNF and IL-6 expression was associated with the presence of Coal-mine dust particles, suggesting a direct role of mineral particles in the cytokine production and development of pneumoconiotic lesions in Coal Worker's Pneumoconiosis (CWP).

Surprisingly, the TNF-α was not significantly increased by CD exposure. TNF-α indirectly induces pulmonary inflammation in response to particulate by stimulating the excretion of other chemical mediators from the epithelial cells, fibroblast and endothelial cells (Driscoll *et al.,* 1997).

Moreover, Zhai *et al*. (2002) suggested that serum levels of TNF receptors and IL-6 are associated with the fibrotic process of CWP and serum cytokine levels may be correlated with the severity of CWP). Rao *et al.* (2005) found that there was no change in IL-10, Transforming Growth Factor-B (TGF-B), or TNF-mRNA levels in BAL cells, likewise, we noted no significant changes in IL-6 mRNA levels of BAL cells after DEP exposure. Data illustrated in Fig. 3 showed a significant increase in nuclear proteins measured in the nuclear extract of AM in CD-exposed rats at doses of 20 and 40 mg/rat. This result suggests that CD enhances nuclear protein expression. Further investigations are required to know the mechanism by which CD enhances nuclear protein expression. Collectively, these findings highlight the necessity for further validation prospective studies and the assessment of novel molecules (Strieter *et al.*, 2004) to serve as diagnostic and prognostic tools as well as markers of the disease activity and severity. The recent application of massive genome screening tools such as DNA microarrays in the respiratory research field (Tzouvelekis *et al*., 2004) has led to an increase rate of discovery of genes involved in the disease initiation and progression. Thereby, the next challenge arising from the emerging of hundreds of candidate biological markers is the application of the genome discoveries in the clinical setting with the use of tissue microarrays (Rubin, 2001) in order to establish their diagnostic, prognostic and therapeutic importance and lead to a better understanding of the biological characteristics of ILDs. Alteration of pulmonary xenobiotic pathways was determined by monitoring the protein levels and activities of P-450 isozymes (CYP1A1 and Cytochrome P450281 (CYP2B1), glutathioneS-transferase (GST), and NADPH:quinone oxidoreductase (QR), (Jane et al., 2003)

## Conclusion

From our results we can conclude that CD exposure induces pulmonary inflammation, increases the production of IL-1β, and increase AM nuclear proteins that may be related to CD-mediated suppression of CYP1A1 induction

## Recommendations

As a result of our investigation we can recommend that released Cytokine may be identified as a reliable prognosticator of the disease progression.

Analysis of BALF and AM may be used as a relevant indicator of inflammatory effects of CD on lungs before clinical symptoms of toxicity appear.

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