PULMONARY EXPOSURE TO FOREIGN PARTICLES AS CYTOCHROME P450 (CYP1A1) SUPPRESSOR

By

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SUMMARY

Exposure to some dusts, such as respirable coal dust (CD) is a common cause of lung injury and pneumoconiosis in human and animals. Lung is a more susceptible organ for exposure to polycyclic aromatic hydrocarbons (PAHs), such as tobacco products. The pulmonary toxicity and carcinogenicity of PAHs depend upon metabolic activation by cytochrome P4501A1 (CYP1A1), which is inducible by its own substrates and can activate the PAHs into reactive metabolites producing DNA nucleotide adducts. Preliminary results showed that mixed exposure to both CD and PAHs reduced the metabolic activities of CYP1A1 in rats, we have examined the hypothesis that CD alters the localization and metabolic activity of CYP1A1 in the particle-exposed lung of ovine model. Therefore, the right apical lung lobes of lambs were instilled with 500 mg respirable CD (n=4) or saline (n=5) using a bronchoscope. The lambs were intraperitoneally injected with 50 mg/kg of the model PAH, beta-naphthoflavone (BNF), on day 53 and 54 post-exposure (to induce CYP1A1) and euthanized on day 56.

By immunofluorescence, the expression of CYP1A1 was significantly reduced within the alveolar septum. The CYP1A1-dependent 7-ethoxyresorufin-O-dethylase (EROD) activity was significantly decreased in the pulmonary microsomes from CD-exposed lobes. By Western blot analysis, the CYP1A1 apoprotein was reduced in CD-exposed lobes compared to control. These findings in sheep model support the hypothesis that respirable CD modifies the induction and pulmonary localization of CYP1A1 protein.

INTRODUCTION

Preliminary data from experiments on rats suggest suppression of CYP1A1 induction and its dependent enzymatic activity (EROD) by pulmonary exposures to respirable CD (Ghanem *et al*, 2003). In addition, CD particles suppress the enzymatic activity (PROD) of the CYP2B1 isoform in rat lungs (Ghanem *et al,* 2003). However, the comparability of rat and human pulmonary responses to respirable particles of low toxicity has been questioned (ILSI, 2000). Activity, quantity, and localization of CYP1A1, the CYP isoform which activates some polycyclic aromatic hydrocarbons (PAHs), such as those in cigarette smoke, to produce DNA-binding carcinogenic intermediates was studied by Bjelogrlic *et al*, 1993 and Liang *et al,* 2003. In addition, we have studied the activity of CYP2B. CYP2B is the major constitutively expressed CYP member in sheep lung, which is analogous to CYP2B1 in rats (Williams *et al,* 1991). Lambs were selected as a model because the ovine response to respirable crystalline silica, another cause of pneumoconiosis, was similar to that observed in humans (Begin *et al,* 1989; Larivee *et al,* 1990). Ovine lungs are also large, which permits directed exposure and the use of internal control lobes, an important advantages in an outbred species.

The sheep coding region of CYP1A1 has an 85% homology to human CYP1A1 (Hazinski *et al,* 1995). Therefore, sheep appear to be an appropriate model to study the effect of xenobiotics, such as PAHs, on CYP1A1-associated carcinogenic pathways. The aim of this study is to use the lamb as a non-rodent model to establish the relationship between pneumoconiosis, caused by CD deposition in ovine lungs, and alterations in xenobiotic metabolism.

MATERIALS AND METHODS

Sheep

Nine 3-month-old Katahdin crossbred castrated male lambs weighing 17-30 kg were used in this study. The lambs were housed in the Food Animal Research Facility (FARF) of West Virginia University. Lambs were fed Alfagreen Supreme Dehydrated alfalfa pellets (containing 17% crude protein, 1.5% crude fat and 30% crude fibers), with *ad libitum* supply of water. The lambs were kept for 3 weeks prior to exposure for acclimatization. During this period, they were examined and all parameters were within normal range.

To assure parasite-free lambs, ivermectin was injected subcutaneously 6 and 3 weeks before instillation.

Experimental Design:

Lambs were randomized into a CD-exposed and a control group using a randomizing program (www.randomized.com). The lambs were exposed in groups of 2-3 lambs / day over an eight day period. The lambs were sacrificed eight weeks after exposure. All lambs were subcutaneously injected with 10 mg/kg Tilmicosin antibiotic (Mycotil, Eli Lilly, Indianapolis, IN) one day after instillation of the final lamb as a prophylaxis against pulmonary infections. Three and two days before sacrifice, lambs were intraperitoneally (IP) injected with 50 mg/kg BNF, to induce pulmonary CYP1A1.

Preparation of Particle Suspension

CD particles (< 5 micrometer in diameter, 500 mg/lamb) were heat sterilized for 2 h at 160 °C. The particles used in this study contained 0.34 % total iron of which 0.119 % was surface iron. The CD suspension was prepared by addition of 15 ml sterilized saline to 500 mg CD and vortexed. The entire volume was used for intrapulmonary instillation. In control animals, 15 ml saline alone was instilled. The suspension was drawn into a sterile syringe attached to a 1 mm diameter polyethylene tube and inserted into an endoscope (Jorgensen Laboratories Inc., Loveland, CO). The whole amount was instilled in the right apical lobe. In the control group, only 15 ml of the sterile saline was instilled into the same lobe under the guidance of the fiberoptic bronchoscope.

Intratracheal Instillation of Particles Using Flexible Fiberoptic Bronchoscope

Lambs were anaesthetized by intramuscular injection of ketamine hydrochloride (Keta-ject, Phoenix Laboratories Inc., St. Joseph, MO) 11 - 15 mg/kg and xylazine hydrochloride (Xyla-ject, Phoenix Laboratories Inc., St. Joseph, MO) 0.22 mg/kg.

A fiberoptic bronchoscope (Jorgensen Laboratories Inc., Loveland, CO) with a 5-mm external diameter was inserted through a speculum into the trachea and the CD suspension was instilled into the lumen of the bronchus of the right apical lobe. Importantly, in sheep, the right apical lobe is divided into 2 segments, the cranial segment and the caudal segment, each one receives a separate bronchus from the mainstem right tracheal bronchus (Getty, 1975). In our instillation procedure, the CD was instilled in the mainstem

tracheal bronchus so that both segments were exposed. After instillation, lambs were kept on the right side to allow settling of particle suspension within the instilled lobe. To facilitate anesthetic recovery, yohimbine hydrochloride (Yobine, LLOYD Laboratories, Shenandoah, OH) was injected IV (0.2-0.4 mg/kg) after the lambs were placed in the recovery stall.

Lamb Necropsy

Lambs were euthanized by intravenous injection of Sodium Pentobarbital (Sleepaway®, Fort Dodge Animal Health, Fort Dodge, Iowa) 26 mg/lb. The entire lung was weighed and the right tracheal bronchial lobe and the left apical lobe were separately weighed. A piece of lung tissue weighting approximately 10 % of each lobe was excised for microsome preparation and the other 90 % was fixed by airway perfusion with a volume of 3 ml of 10 % neutral buffered formalin per gram lung to prepare of 4- µm sections for immunofluorescence .

Preparation of Lung Microsomes

Microsomes were obtained by differential centrifugation method as previously described (Flowers and Miles, 1991; Ma *et al,* 2002). These microsomes were used for measuring the CYP1A1 and CYP2B1-dependent enzymatic activities (EROD and PROD, respectively). In addition, lung microsomes were subjected to electrophoresis to determine the CYP1A1 protein by Western blot analysis.

Determination of the Total Lung Proteins

The total amount of protein in the microsomes was determined by the bicinchoninic acid (BCA) method as previously described (Smith *et al,* 1985 ;Ma *et al,* 2002) using the BCA protein assay kit (Pierce, Rockford, IL) in a spectra Max 250 Spectrophotometer (Molecular Devices Corporation, Sunnyvale, California). Bovine serum albumin was used for the standard curve. Protein was measured as mg/ml.

Determination of CYP1A1- and CYP2B-Dependent Enzymatic Activities (EROD and PROD)

Spectrofluorometric assays for measuring EROD and PROD metabolic activities were performed as previously described (Burke *et al,* 1985; Ma *et al,* 2002) using a luminescence spectrometer model LS-50 (Perkin-Elmer, Norwalk, CT) and resorufin (Sigma, St.

Louis, MO) as the standard. EROD and PROD activities were expressed as picomoles of the produced resorufin per minute per milligram microsomal protein (pmol/min/mg protein) as previously described (Ma *et al,* 2002).

Immunofluorescence Double Labeling

Fixed tissues were processed at the day of collection to preserve antigenicity. A representative slide from each lobe was used for immunofluorescent detection of CYP1A1 and the alveolar type II cell (AT-II) marker, cytokeratins 8/18. Slides containing 4-micrometer-thick tissue sections were heated in the oven at 60 °C for 15 minutes. Deparaffinization and rehydration were routinely conducted by sequential immersion in xylene and alcohol. Antigen availability was maximized by heating the slides in a microwave with 0.01M EDTA, pH 8.0. Non-specific binding was blocked by incubating the slides with 5% bovine serum albumen (Sigma-Aldrich Co.) (in PBS) for 10 minutes followed by incubation with 5% pig serum (Biomeda Corporation, Foster City, CA) for 10 minutes at room temperature. The primary antibodies were a polyclonal rabbit CYP1A1 antibody (Xenotech, Kansas City, KS) diluted 1:5 with PBS and a polyclonal Guinea pig anti-cytokeratins 8/18 (Researech Diagnostic Inc., Flanders, NJ) diluted 1:50 with PBS. Rabbit serum was applied as a negative control. After 48 h incubation at room temperature, the slides were incubated for additional 2 h at 37 °C. The slides were then thoroughly washed to remove un-conjugated primary antibodies and the secondary antibodies were dropped onto the slides. The secondary antibodies mixture consisted of an equal volume of an Alexa 594-conjugated goat anti-rabbit antibody (Molecular probes, Eugene, Oregon) diluted 1:20 with PBS and a FITC-labeled, donkey anti-Guinea pig IgG (Research Diagnostic Inc., Flanders, NJ) diluted 1:50 with PBS. The slides were incubated with the secondary antibodies for two h in the dark at room temperature. The slides were visualized using a fluorescent photomicroscope (OlympusAX70, Olympus American Inc., Lake Success, NY) and images were captured using the 40x objective (an area of 34466.1 μm^2) and a Quantix cooled digital camera (Photometrics, Tucson, AZ) with QED camera plugin software (QED Imaging, Inc., Pittsburgh, PA). Five images were captured per slide from the proximal alveolar (PA) regions, where most of the CD particles tend to localize near the terminal bronchioles and alveolar ducts.

Morphometry

Immunofluorescence morphometric analysis using Metamorph software (MetaMorph, Universal Imaging Corp., Downingtown, PA) was conducted on digital images. The area of CYP1A1 expression was quantified in AT-II and alveolar septal cells that did not contain AT-II markers [non-type II cells (NT-II] and entire alveolar septum.

Western Blot Analysis

The amount of CYP1A1 apoprotein in lung microsomes was determined by Western blot as previously described (Ma *et al,* 2002) with minor adaptation. A Novex Tris glycine gel with 15 small wells, (Invitrogen Corporation, Carlsbad, CA), and 30 µg of microsomal proteins were subjected to SDS gel electrophoresis for 90 min at 120 volts followed by transfer to a nitrocellulose membrane (blotting) for another 90 minutes at 25 volts. Liver microsomes of BNF-treated rat (Xenotech, Kansas city, KS) were used as a positive control. Non-specific binding was blocked by incubating the membrane with a 5% solution of dry milk in tris-buffered saline (TBS) for 1 h at room temperature. The protein bands were identified by using a primary polyclonal rabbit anti- rat CYP1A1 antibody (Xenotech, Kansas city, KS) for overnight incubation at 4 °C. The membranes were washed and incubated for 1 h with a HRP (horse radish peroxidase)-conjugated goat anti-rabbit IgG (Santa Cruz Biotech. Inc., Santa Cruz, CA) at room temperature. Super RX Fuji Medical X-ray film was then exposed to the membranes at room temperature. Band intensity was scanned by Eagle Eye II with Eagle Sight software (Strategene, La Jolla, California). The density was measured using ImageQuant software version 5.1 (Amersham Pharmacia Biotech, Piscataway, NJ). After quantification, the data were presented as a percentage of the positive control.

Statistical Analysis:

All analyses were performed with SAS version 8.2 the model used was two factor repeated measures analysis of variance. All pairwise comparisons were performed using a pooled variance estimate. All results were considered statistically significant at $p < 0.05$ (Ghanem et al, 2003)

iii- CYP1A1 Expression in NT-II

The area of CYP1A1 expression in NT-II is significantly diminished (80.59%) in lambs exposed to CD and BNF compared to BNF alone ($p=0.0015$) (Fig. 4.) or to the left unexposed lobes of the CD-exposed sheep ($p<0.0299$) (Fig. 3E).

2- 7-Ethoxyyresorufin-O-Deethylase (EROD) Activity

The CYP1A1-dependent enzymatic activity (EROD), was significantly reduced (31.57%) in lambs exposed to CD and BNF compared to those exposed to BNF alone ($p=0.0166$) or to the uninstilled lobe (left lobe) ($p=0.0265$) (Fig. 4A).

3- 7-Pentoxyresorufin-O-Deethylase (PROD) Activity

The CYP2B-dependent enzymatic activity (PROD) was significantly lowered in CD-exposed lambs with BNF compared to control ($p=0.042$) (Fig. 4B).

4- Western Blot Analysis

CYP1A1 protein bands of right exposed lobes and left unexposed lobes were quantified and expressed as percentage of positive CYP1A1 control. The CYP1A1 apoprotein measured in lung microsomes by Western blot was reduced by 32.46% in lambs exposed to CD and BNF compared to control and reduced by 9.49% in the CD-exposed lobes compared to the control unexposed lobes (Fig. 5).

DISCUSSION

In this study, we investigated the modifying effect of respirable CD particles on CYP1A1 induction in BNF-exposed sheep. The ovine model was selected because the response of ovine lung to respirable particles, such as quartz, is similar to the human response, particularly in cellular cytotoxicity (Larivee *et al,* 1990).

The results indicate that CD significantly inhibited CYP1A1-dependent metabolic activity (EROD) in lung microsomes (Fig. 4). This effect was localized only in the lobes that were instilled with CD (right lobes). However, in the left lobes, which were not exposed to CD, there was no change in the activity. This suggests that CD exposure inhibited CYP1A1 metabolic activity locally and the effect did not extend to include unexposed lung lobes.

To assess the effect of CD exposure on CYP1A1 induction, the CYP1A1 protein was measured by Western blot. The amount of

CYP1A1 protein measured by Western blot was reduced, albeit not significantly, in the CD-exposed right lobes compared to the saline-exposed lobes and compared to unexposed lobes. Although the reduction was not significant, the general trend seemed to be suppressive. This result suggested that CD exposure in sheep not only inhibited CYP1A1-dependent EROD activity but inhibited the CYP1A1 protein expression as well.

The suppressive effect of the CD on CYP1A1 induction in sheep lungs was further demonstrated by immunofluorescence double labeling. By using this technique, the cellular expression of CYP1A1 was investigated within the alveolus. We used marker for cytokeratins 8/18, which are cytoskeletal proteins highly expressed in primitive epithelial cells, to recognize the AT-II (Kasper *et al,* 1993) in the lung alveolus. Accordingly, the cellular components of the stained lung tissue section were divided into two distinct populations using indirect immunofluorescence with a primary anti-cytokeratins 8/18 antibody and a green FITC-labeled secondary antibody. One population stained distinctively green and those were AT-II (Fig. 1A). The others did not stain green and were designated as NT-II. The area of CYP1A1 expression measured in NT-II, where no green fluorescence was visualized, was significantly higher than that area in AT-II. This result suggests that in the alveolus, AT-II are not the major sites of CYP1A1 induction and NT-II are important sites of CYP1A1 induction. In addition, there is a general deficit in the literature regarding the localization of inducible CYP1A1 in ruminant lungs. The majority of literature in ruminant CYPs concentrates on the inducibility of CYP1A1 in the liver of goat (Kasper *et al,* 1993) or cattle (Skalova *et al,* 2001) and this study is the first, to our knowledge to report the distribution pattern of CYP1A1 in sheep lungs.

The CD instillation in sheep reduced the area of CYP1A1 expression in AT-II, NT-II and the entire alveolar septum (Fig. 1D). Therefore, one mechanism of suppression of BNF-induced CYP1A1 by CD exposure appears to be inhibition of CYP1A1 expression in different alveolar septal cells. These results are not surprising and are comparable to those seen in rats exposed to CD in preliminary data (Ghanem *et al,* 2003). While increased size (hypertrophy) and number (hyperplasia) of AT-II were not significant in this study, they were increased numerically which is consistent with preliminary studies in rat model (Ghanem *et al,* 2003). The

area of CYP1A1 expression in AT-II, relative to the total area of AT-II (proportional CYP1A1 expression, Fig. 3D) showed a significant reduction suggesting that the new hyperplastic AT-II do not express CYP1A1 in proportion to their number and size. This result suggested that CD exposure led to production of a new population of AT-II with decreased CYP1A1 expression. The mechanism of downregulation of CYP1A1 associated with cellular proliferation should be further investigated. However, in the rat liver with hyperplastic nodules, induced by diethylnitrosamine and partial hepatectomy, the total amount of microsomal CYP enzymes was reduced 50% compared to the control (Degawa *et al,* 1995). Moreover, the inducibility of CYP1A by inducers decreases slightly in the rat liver bearing hyperplastic nodules (Degawa *et al,* 1995). All of these previous studies suggest that CYP protein is downregulated in proliferating cells - - a finding which is consistent with the downregulation of CYP1A1 induction and CYP2B in our study of CD-exposed lambs.

Along with CYP1A1 activity, the activity of another CYP isoform, CYP2B, was measured. CYP2B is the major constitutive isoform of CYP family in sheep lungs (Williams *et al,* 1991). The CYP2B-dependent enzymatic activity (PROD) showed a significant diminution in lung microsomes prepared from lung of sheep exposed to CD and BNF compared to BNF alone (Fig. 4B.). This result suggested that, CD not only inhibited the activity of BNF-induced CYP1A1 in sheep lung, but also the activity of another CYP isoform, CYP2B, which is constitutively expressed in sheep lungs.

The response of the rat lung to high concentrations of respirable particles is of debatable relevance for human risk assessment (ILSI, 2000). In particular, the alveolar epithelial cell proliferation seen in response to respirable particles is more pronounced in rats than in primates (Nikula *et al,* 1997). For that reason, an ovine model was used to investigate the effects of CD on CYP1A1 induction in the lung. We found that CD suppressed CYP1A1 induction. In addition, the activity of the major constitutive isoform of CYP in the ovine lung, CYP2B, was suppressed by respirable CD. This is similar to suppression of CYP2B1 previously noted in the particle-exposed rat lung (Ma and Ma, 2000).

In conclusion, CD suppressed the activity of both CYP1A1 and CYP2B in a non-rodent, ovine model. This is consistent with

the inflammation-associated downregulation of many CYP isoforms in rodent model systems.

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FIGURE LEGENDS

Fig. 1. Immunofluorescent images showing the suppression of CYP1A1 expression in the alveolus after CD and BNF exposure. A. Immunofluorescent imaging of the ovine lung treated with BNF alone showing the general distribution pattern of cytokeratins 8/18 (green) which is expressed primarily in AT-II that are cuboidal in shape and located at the corners of alveolar septa. Cells that do not express cytokeratins 8/18 are not green and are usually elongated cells (NT-II) B. Immunofluorescent imaging of the ovine lung treated with BNF alone showing the general distribution pattern of CYP1A1 (red). C. Dual immunofluorescence for CYP1A1 (red) and cytokeratins 8/18 (green) in the uninstilled left lobe of a BNF-injected sheep showing that most of the CYP1A1 is localized in NT-II that do not express cytokeratins 8/18. CYP1A1 expression in AT-II is indicated by a yellow color. D. Using dual immunofluorescence, the red immunofluorescence for CYP1A1 is reduced throughout the alveolar septum in the CD-exposed right lung lobe. Bar=20 µm.

Fig. 2. Morphometric quantification of the cytokeratins 8/18 expression in alveoli of CD exposed vs. control sheep. The area of cytokeratins 8/18 expression is higher, albeit not significantly, in the

CD-exposed right lung lobes of lambs treated with CD and BNF compared to the left unexposed internal control lobes—and compared to right lobes of control BNF-treated lambs. Results represent means ± SE, n=5 in the saline-exposed group and 4 in the CD-exposed group.

Fig. 3. Morphometric quantification of CYP1A1 expression in the ovine pulmonary alveolus. A. Morphometric analysis showing the distribution of CYP1A1 in ovine lung. The area of CYP1A1 expression in NT-II is significantly larger compared to CYP1A1 area in AT-II of left and right lung lobes. B. The area of CYP1A1 expression in the alveolus of the CD-exposed right lung lobe of lambs treated with BNF is significantly lower (74.14%) than with BNF alone and is reduced by 65.3 % compared to the left unexposed lobes of CD-exposed sheep. C. The area of CYP1A1 expression in AT-II was reduced, albeit not significantly in CD-exposed lobes compared to controls. D. The proportional CYP1A1 expression in AT-II of BNF-induced lambs is significantly reduced in the CD-exposed right lung lobe compared to the left unexposed lobes and the right lobe of lambs receiving only BNF. E. Morphometric quantification of the area of CYP1A1 expression in NT-II. In BNF treated lambs, the area of CYP1A1 expression in NT-II is significantly lower in the right CD-exposed lobes compared the left unexposed lobes of the same lambs or the unexposed lung of lambs receiving BNF alone. Results represent means ± SE, n=5 in the saline-exposed group and 4 in the CD-exposed group. * indicates significant difference at $p<0.05$. ** indicates significant difference at $p<0.001$.

Fig. 4. Effect of CD exposure on the EROD and PROD activity in BNF-exposed lambs. A. In BNF-induced lambs, EROD is significantly reduced in the CD-exposed right lung lobes compared to the left unexposed lobes or the lung of lambs receiving BNF alone. B. PROD is significantly reduced in lambs exposed to CD and BNF compared to lambs with BNF alone and is reduced 53.45 %, albeit not significantly, in CD-exposed lobes compared to the left unexposed lobes. Results are means ± SE, n=5 in the saline-exposed group and 4 in the CD-exposed group. * indicate Significant difference at $p<0.05$.

Fig. 5. Western blot analysis showing the reduction of CYP1A1 immunoprotein by CD exposure. A. western blot for the left unexposed lobes. B. Western blot for the right exposed lobes. C. Graphical representation of the amount of CYP1A1 protein measured by Western blot. The letter c above the lane means

control. MW means molecular weight standard. Lanes from 1 to 4 are for saline-exposed lambs (control) while lanes 6 to 10 are for CD-exposed lambs.

Figure 1

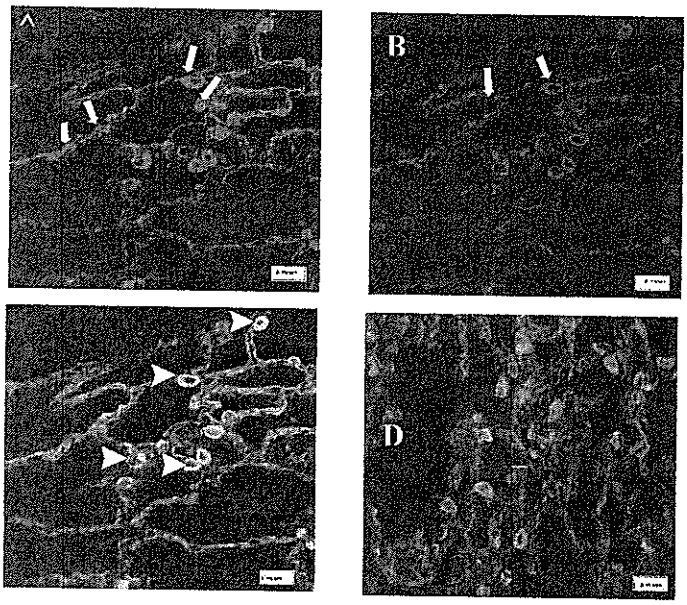


Figure 2

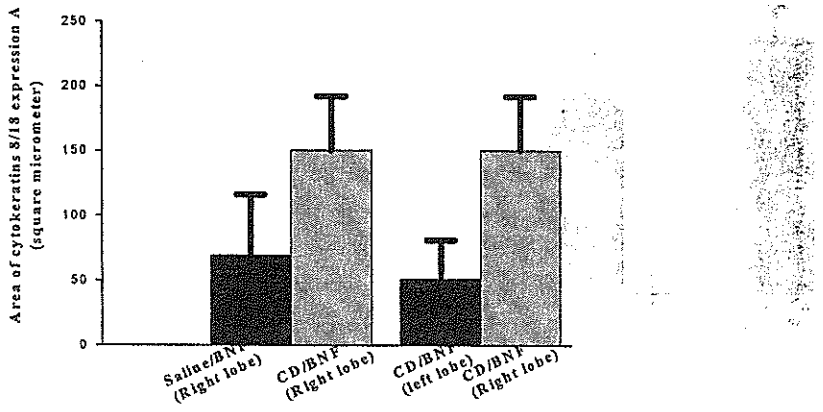
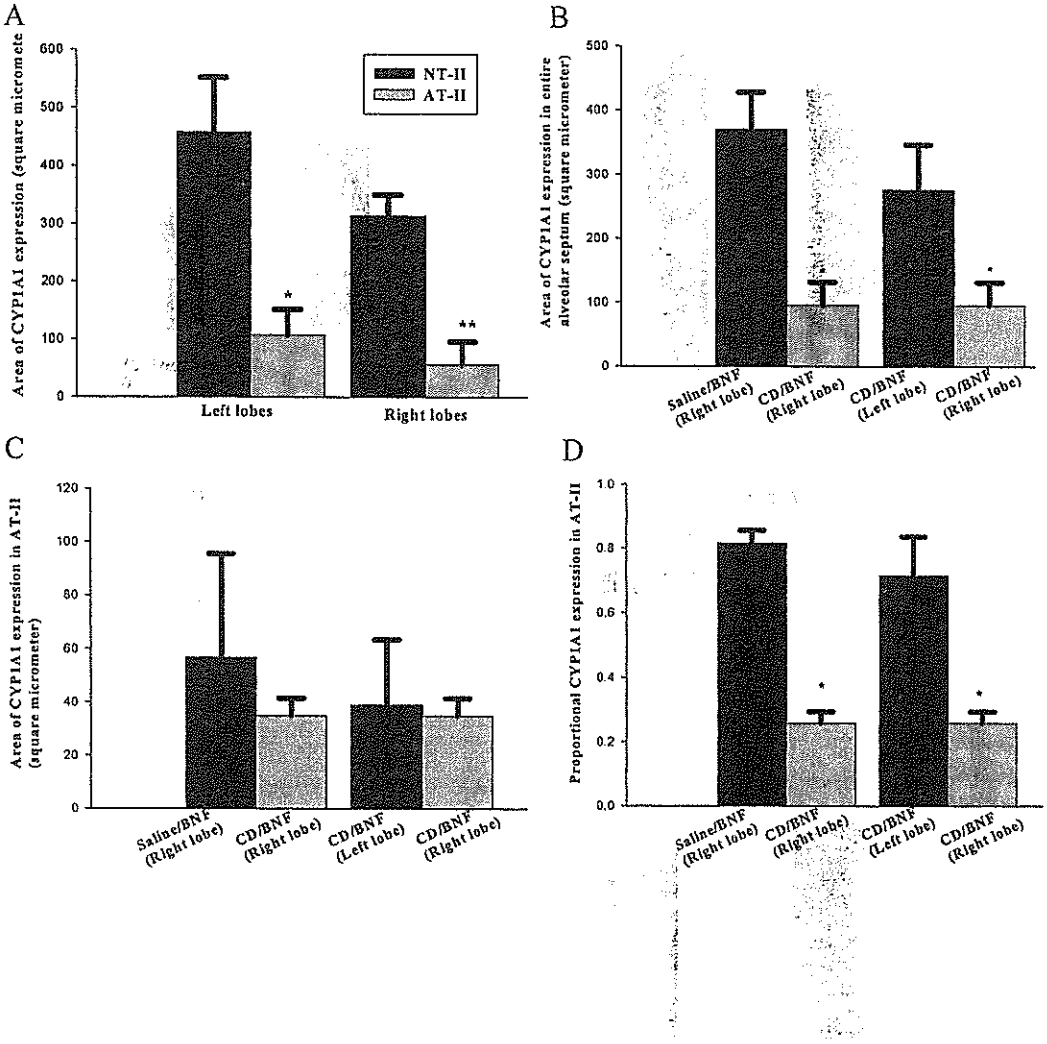


Figure 3



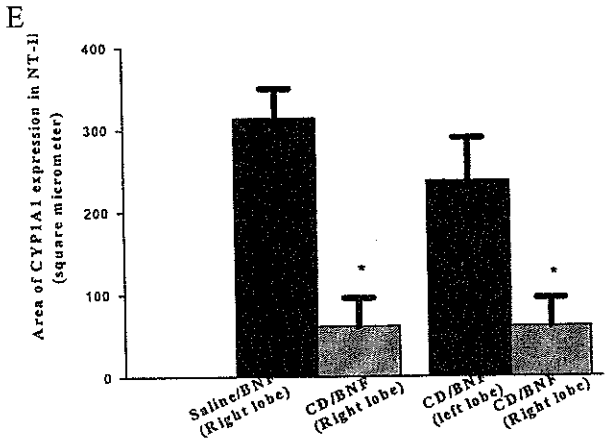


Figure 4

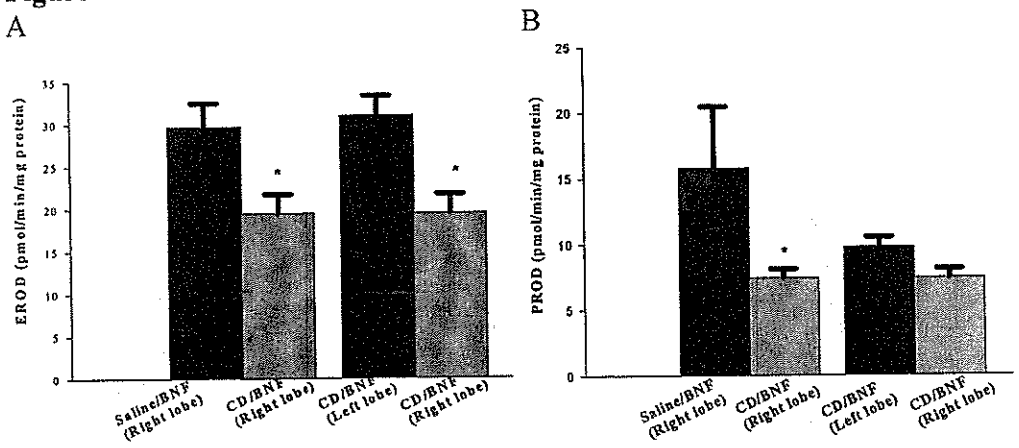
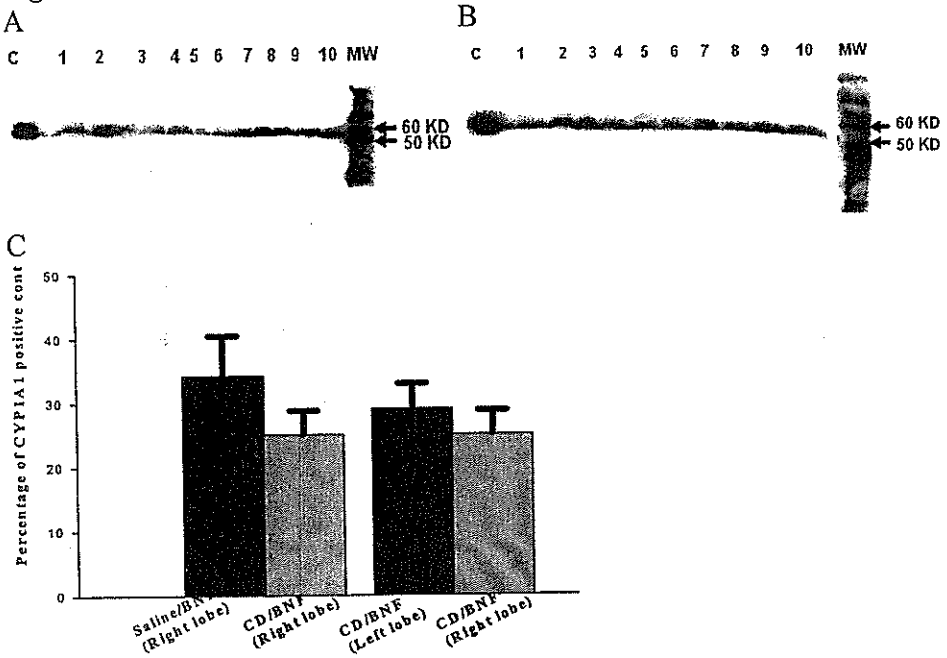


Figure 5



الملخص العربى

التعرض الرئوى للاتربة الغريبة كمثبط لانزيم السيتوكروم CYP1A1

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قسم طب الحيوان[1] و قسم الباثولوجيا الاكلينيكية[2] بكلية الطب البيطرة بمشتهر

قسم الوراثة والبيولوجيا المتطورة[3] بجامعة ويست فيرجينيا بالولايات المتحدة الامريكية

ان التعرض لبعض الاتربة مثل اتربة الفحم يتسبب فى التهاب وتلف انسجة الرئة فى الانسان والحيوان. فالرئة ايضا هى العضو الاكثر تعرضا للمواد الهيدروكربونية الموجودة فىمنتجات التبغ. ويعتمد التسمم الرئوى و السرطانات الرئوية على النشاط التمثيلى لانزيم السيتوكروم CYP1A1 والذى ينتج عن طريق هذة المواد وفى نفس الوقت يقوم بتحويلها الى مواد اكثر نشاطا حيث تتفاعل مع نيوكليتيدات الحامض النووى DNA محدثة خلل. تشير النتائج الاولية ان التعرض المتزامن لاتربة الفحم والمواد الهيدروكربونية يقلل النشاط التمثيلى لانزيم السيتوكروم CYP1A1 ولذلك فان هذا البحث يدرس تاثير اتربة الفحم على تغييرات الاماكن المعتادة المتواجد بها انزيم السيتوكروم CYP1A1 وكذلك نشاطة التمثيلى فى الاغنام. ولذلك تم تعريض الفص الامامى من الرئة اليمنى للاغنام الى 500 مجم من تراب الفحم باستخدام المنظار الشعبى وفى اليوم ال 53 و 54 بعد التعرض للاتربة تم حقن الاغنام ب 50 مجم / كم من BNF وذلك لتنشيط تكوين السيتوكروم CYP1A1 يليها ذبح الغنام فى اليوم 56 بعد التعرض الرئوى. لوحظ فى النتائج ان التعرض لاتربة الفحم يؤدى الى نقص معنوى فى انتاج السيتوكروم CYP1A1 فى جدران الحويصلات الهوائية باستخدام الاشعاع المناعى. كما لوحظ نقص معنوى فى النشاط الكيميائى المعتمد على انزيم السيتوكروم CYP1A1 المقاس فى ميكروسومات االخلايا الرئوية . وباستعمال الويستيرن بلوت لوحظ نقصان فى بروتين السيتوكروم CYP1A1 فى الفصوص التى تعرضت للاتربة مقارنة بالكنترول. هذة النتائج تدعم النظرية الافتراضية بان التعرض لاتربة الفحم يؤدى الى تغير انتاج وكذلك اماكن تواجد السيتوكروم CYP1A1 فى الاغنام.

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