IN VITRO AND *IN VIVO* REACTIVITY CHANGES IN GUINEA PIG AIRWAY SMOOTH MUSCLE IN RESPONSE TO CHALLENGES FROM HEAT - TREATED AND UNTREATED COTTON DUST EXTRACT.

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Abstract

As part of an investigation into the effect of the heat detoxification of cotton dust[1] *in vitro* and *in vivo* experiments were carried out to compare the toxicological potential of inhaled aqueous cotton dust extracts (CDEs). The *in vitro* work, carried out on both tracheal spiral and perfused lung preparations, demonstrated a phase of hyperresponsiveness due to the action of both types of dust extract. The hyperresponsiveness manifests itself as a leftward shift in the dose - response curve of the standard bronchoconstrictor. The degree of shift was ten - fold in the case of the untreated dust and one and a half - fold for the heat - treated dust. The hyperresponsiveness was not significantly affected by any of the pharmacological agents (atropine, chlopheniramine, FPL 55712 or indomethacin) except in the case of the TXA_2 antagonist OKY048. Comparisons are made with results obtained from similar work with pure lipopolysaccharides (LPS).

In vivo, using a whole animal restrained plethysmographic method, hyperresponsiveness to the dust extract was developed approximately 1h post exposure. The reactivity change developed in the case of both dust types but the degree to which the change occurred varied, the untreated dust being more able to induce a reactivity change (20%) compared to change of 15% with the heat - treated dust. The result correlates well with the time taken to induce a hyperresponsive change as seen with pure LPS. However, the overall percentage change in reactivity was not as great with the dust extracts, the change being on average 17% compared with a 30% reduction with the pure LPS.

The heat treatment protocol may therefore be regarded as a detoxification process although complete detoxification of the extract does not occur. Although LPS has been shown, both directly and indirectly, to be a major contributor to the induction of hyperresponsiveness in guinea pig airways; other components present in the extracts may also play an important contributory role e.g. tannins, glucans.

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Introduction

Various respiratory symptoms have been observed in workers employed in the processing of organic materials such as cotton , hemp and flax. The air of these work environments is polluted by dusts derived from the processing of the material in question. Symptoms presented include cough, wheeze, sneezing and chest tightness. Chronic exposure to dusts in these manufacturing environments leads to the development of serious respiratory conditions such as byssinosis.

Recent work suggests that gram negative bacterial lipopolysaccharide (LPS) may be an important etiological factor. Cotton bolls are heavily contaminated with gram negative bacteria e.g. *Enterobacter agglomerans.* The processing involved in producing cotton means that these bacteria are lysed and become a part of the respirable fraction of the working environment, along with other components of the plant fibres e.g. tannins and other contaminant organisms such as fungi and yeasts. Rylander *et al*[5] have demonstrated a better correlation between respiratory symptoms and airborne endotoxin levels than with airborne dust levels. Thus any procedure which reduces the LPS/ endotoxin present in the working environment would be of benefit in the reduction of symptoms in cotton workers and a possible benefit in the goal of fully understanding the development of conditions such as byssinosis and organic dust toxicity syndrome (ODTS).

Following a study carried out on the effects of CDE on the reactivity status of the airways, it has been demonstrated that the CDE's, both heat treated and untreated extracts can produce a phase of hyperresponsiveness *in vitro*[1]. The heat treatment carried out on the dust obtained from cotton fibre processing has shown to reduce endotoxin content of the dust by approximately 70%[3]. This represents a large decrease in endotoxin content and hence a theoretical decrease in potential toxicity.

Methods

The cotton used was of the variety MD-51, grown in Stoneville, MS. The cotton dust was prepared and collected as outlined by Rouselle *et al*[4] . The dusts were derived from a batch of normal (control) cotton and one of the control cotton that had been subjected to heat treatment shown to greatly reduce LPS content[3]. We are grateful to Marie - Alice Rouselle for providing the samples.

Extraction procedure

1. Twenty grams (20g) of dust material was added to 200ml of double distilled and deionised water. The water was LPS free.

2. The slurry was stirred and ground thoroughly using a pestle and mortar for at least fifteen minutes.

3. The thick slurry that formed was allowed to macerate overnight at 4^0c - in order to inhibit bacterial growth.

4. The majority of the solid matter was removed by filtration under reduced pressure through a gauze cloth.

5. The dust mat formed was rinsed with 50ml of LPS - free water.

6. The water rinsed from the dust mat was added to the liquid from the crude filtration and centrifuged at 3000g for 10 - 20 mins in order to clarify the extract.

7. The supernatant was decanted and the solid material discarded. The liquid was then filtered under reduced pressure through Whatman no.1 filter paper.

8. The liquid extract was then freeze dried until a light freeze dried powder formed, typical yield being 10% w/w of the original quantity of dust.

9. The freeze dried material was stored at -10^0c or less and reconstituted immediately prior to use by the addition of sterile vehicle.

Drugs used

The drugs used for plethysmography were made up in non - pyrogenic sterile saline (Baxter, Thetford, UK). All drugs used in the *in vitro* procedure were dissolved in Krebs solution.

Atropine sulphate, histamine biphosphate, indomethacin and methacholine chloride were obtained from Sigma Chemicals UK Ltd. FPL55712 was a gift from Fisons Pharmaceuticals, UK. Chlorpheniramine maleate was obtained from Allen and Hanburys, UK. OKY046 w as a gift from Takeda Pharmaceuticals, Japan.

Animals and husbandry

Male Dunkin Hartley guinea pigs of weight range of 350 - 550g were employed throughout. Prior to the experiments the animals were housed in groups of up to six animals in North Kent Plastic aluminium A1 guinea pig holding units (76 x 71 x23 cm) with stainless steel grid floors. The trays beneath the cages were lined with newspaper which was changed three times per week. Tap water, oranges and pelleted food (FDP; Special Diet Services, Bicester, Oxford) were available *ad libitum*. Temperature and humidity were maintained at 21^0c and 50% respectively. A light -dark cycle of 12h was maintained throughout.

***In vitro* experimental procedures**

The animals were sacrificed by cervical dislocation and exsaguination, the trachea and lungs were then removed quickly to warmed Krebs solution. (NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, Na_2HPO_4 1.2, $NaHCO_3$ 25 and glucose 11.1($mmoll^{-1}$)). The excised respiratory organs were cleared of extraneous connective tissue and blood vessels. The lungs were cut into the left and right halves at the bifurcation and attached to a cannula (via the bronchi) using surgical cotton. The lungs were then perfused with Krebs solution using a Watson - Marlow peristaltic pump (rate of flow: 5ml min^{-1}). Their viability was maintained by gassing with a mixture of 95% oxygen & 5% carbon dioxide and the temperature of the Krebs was maintained by using a circulator set at 35^0c. The trachea was cut into two 4cm spirals according to the method of Constantine[6] and suspended in organ baths in Krebs solution and maintained at the same conditions of temperature and oxygenation as the lungs. Both tissue types were left to equilibrate for one hour. Dose - response curves were then obtained to the appropriate bronchoconstrictor prior to adding CD extract to the organ baths (trachea) or perfusing solution (lung). The freeze dried powder was dissolved in the Krebs (25mg of powder $5ml^{-1}$.) The CDE solutions remained in contact with the tissues for one hour. One hour after replacing the bath fluid with Krebs solution, the dose - response curves to the bronchoconstrictor were repeated. Contractions of the trachea were recorded on a Devices MX4 recorder via a Dynamometer UFI isometric transducer. The pressure changes in the lung were recorded on the same recorder via a Bell & Hartley pressure transducer. One lung half was used as a time control to monitor changes of reactivity in normal tissue. The other half contained the test drug within the perfusing solution. Owing to the differing responses of the left and right halves of the lung cross over experiments were carried out i.e. the 'test' was right lung for the first experiment, left for the second experiment etc. The trachea was cut into two halves ; one 4cm spiral acting as a control and the other as the test tissue (containing the relevant pharmacological tool compound).

Student's t - test was applied to all points of the dose - response curve to determine the significance of any changes seen.

I*n vivo* procedure

Exposures

The guinea pigs were placed in an exposure chamber (volume $0.4m^3$) for exposure to either nebulised i) 0.9% sterile saline solution, ii) heat treated CDE or iii) untreated CDE (both at a concentration of 12mg ml^{-1} in sterile saline) for ten minutes. For exposure a Hudson nebuliser was used with air at a pressure of 15 p.s.i. This apparatus produced an aerosol with greater than 60% of particles having a MMAD of 5µm or less.

Procedure

All animals used in the *in vivo* procedure were trained for use in the plethysmograph according to the method of

Griffiths-Johnson *et al* (which also describes the determination of sGaw)[7]. Firstly, a baseline sGaw value was obtained for the response to the bronchoconstrictor methacholine (exposure to a 10µg ml^{-1} solution occurred for 1 min using a Wright nebuliser operating at 20 p.s.i.). sGaw was measured 5min prior to exposure to the bronchoconstrictor and then also measured at 2, 5, 10 & 15 min post - exposure. The post - exposure sGaw divided by the baseline sGaw gave a percentage value for the initial reactivity (IR). Twenty four hours later, the guinea pigs were exposed to either saline, heat treated CDE or untreated CDE as described above then at the given time points 5,10,15,30,45,60,120 and 180 min post exposure the bronchoconstrictor challenge was repeated and a second reactivity value was calculated (NeR) and the change in reactivity value was therefore calculated as IR - NeR. The dose of methacholine given was judged to induce an IR of 30-40% so that decreases and increases in reactivity were detectable. Groups of six to eight animals were used for each exposure.

Results

In vitro

Following gaining the initial dose response to methacholine both lung and tracheal preparations were hyperresponsive 1hr post CDE extract exposure[1]. A ten - fold leftward shift in ED_{50} occurred in the case of the untreated CDE and an approximately 1.5 fold leftward shift in the case of heat treated CDE. These shifts were approximately the same for both preparations (Figures 1 and 2). The anti - histamine (chlorpheniramine) has no significant ($p<0.05$) effect on the hyperresponsiveness *in vitro.* The same result is seen with the anticholinergic (atropine), the LTD_4 blocker (FPL 55712) and the prostaglandin synthetase inhibitor (indomethacin- figures 3&4). Only the Thromboxane A_2 antagonist (OKY048 - figures 5&6) was able to produce any significant change. Each of the compounds had been previously shown to antagonise and/or block their relevant receptor and/or enzyme using specific agonists/ substrates that produced a measurable pharmacological marker constriction.

In vivo

Following exposure of the guinea pigs to a nebulised solution of the heat treated and non treated CDE, changes in the response (sGaw) of the airway to inhaled methacholine were observed (Fig 7). An enhanced bronchoconstriction (hyperresponsiveness) was noted beginning about 15 min post exposure. The degree of change was greater in the case of the non treated dust but not significantly ($p<0.05$) different from that seen after exposure to the heat - treated dust. the effect was transitory and the sGaw began to recover towards baseline after the 1hr peak constriction.

Discussion

The results obtained from this series of experiments bring to light much information about dust extracts and their potential toxicity. Dust extracts whether heat - treated or untreated have the potential to cause direct bronchoconstrictio[n2] as well as a change in reactivity both *in vitro* and *in vivo*. The benefit of the heat treatment undertaken is difficult to analyse using an acute model in one animal species. The i*n vitro* results suggest that some benefit would be derived from the detoxification process as the degree of reactivity change lowered and this suggests a lesser impact on normal respiratory parameters. Exposure to the heat - treated CDE over a long term could point to a considerable benefit in terms of respiratory health but again it is difficult to assess whether this would be a true benefit, using this paradigm as the only marker. *In vivo* the reactivity change seen is not significantly different for both types of CDE. There could be several reasons for this, for example, although the apparatus used to measure sGaw is very sensitive (enhanced by computing technology accuracy and sensitivity) it may not be sensitive enough to dissect the effects of the two CDEs *in vivo. In vivo* systems have the added 'complication' that whole animals have compensatory and defence mechanisms that counter the effects of foreign matter. It should also be considered that there may be no difference in the toxicological potential of the two dusts. A further investigation is warranted. The most useful experiments would be to extend the study across a range of species to get a better overall picture of the true toxicity of the dusts. A chronic animal study is also needed so that comparison can be made with long term workplace studies to validate animal models.

The investigation of mechanism draws some parallels with LPS studies already undertaken. LPS's direct constrictor effect and the hyperresponsiveness induced by LPS have been significantly attenuated by TXA_2 antagonists, this suggests that the production of thromboxanes may be particularly important in the change in reactivity caused by both dusts and the pure LPS. The heat detoxification of the dust lessens the degree of hyperresponsiveness compared to the untreated dust; that heat - treatment reduces the LPS content by 70% suggests that LPS is a significant though not sole contributor to the effects of the inhaled CDE. There arises the consideration of whether a further reduction in CDE LPS content would lead to a further decrease in toxicity or whether there are other components present in CDE which also affect airway reactivity. Recent work carried on the detoxification of LPS[8] suggests that removal of lipid A significantly reduces the toxicity of LPS insofar as when lipid A is absent no significant change in bronchial reactivity can be seen either *in vitro* or *in vivo.* Unfortunately this problem cannot be truly resolved until the question of how LAL results and biological activities of samples may be compared with confidence. Further

investigations into the nature of reactivity changes involved with other components present in dust extract may be of some value in answering part of this problem.

While the qualitative pattern seen in the development of bronchoconstriction with CDE is very similar to that seen with pure LPS, the multicomponent nature of CDE makes facile comparisons misleading. However, the detoxification of CDE and hence LPS can be considered to be an important step in understanding and analysing the pulmonary toxicity of cotton dust.

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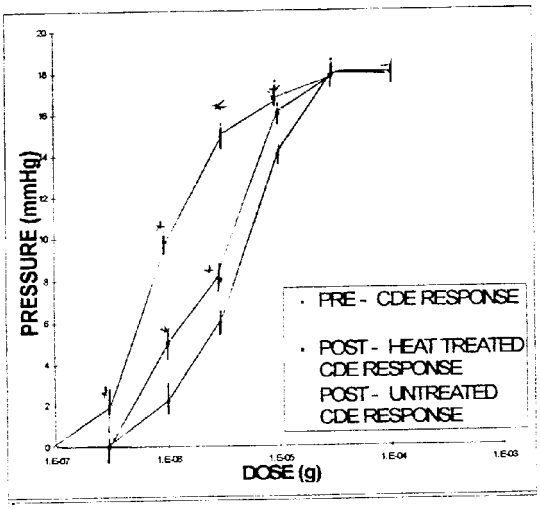

Figure 1. The effect of heat-treated and untreated CDE on pulmonary reactivity to methacholine. *indicates $p<0.05$. n=6. Students t-test.

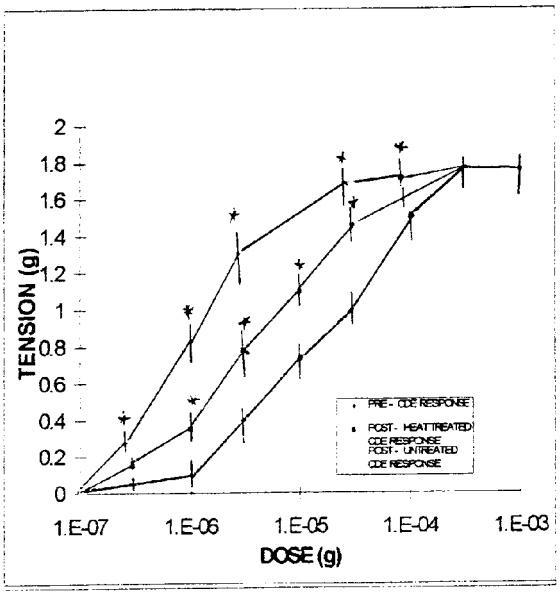

Figure 2. The effect of heat-treated and untreated CDE on tracheal reactivity to methacholine. *indicates $p<0.05$. n=6. Students t-test.

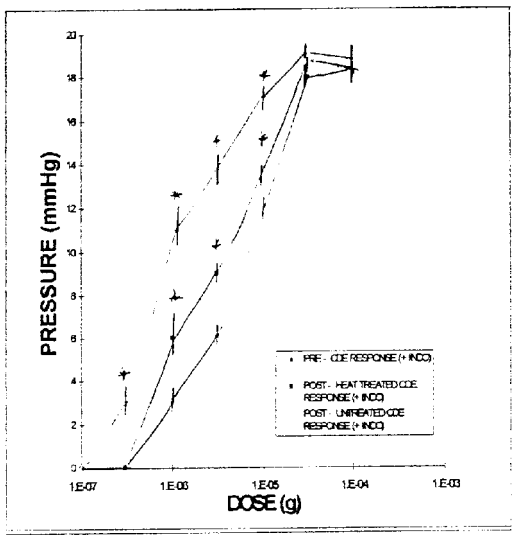

Figure 3. The effect of heat-treated and untreated CDE in the presence of indomethacin on pulmonary reactivity to methacholine. *indicates $p<0.05$. n=6. Students t-test.

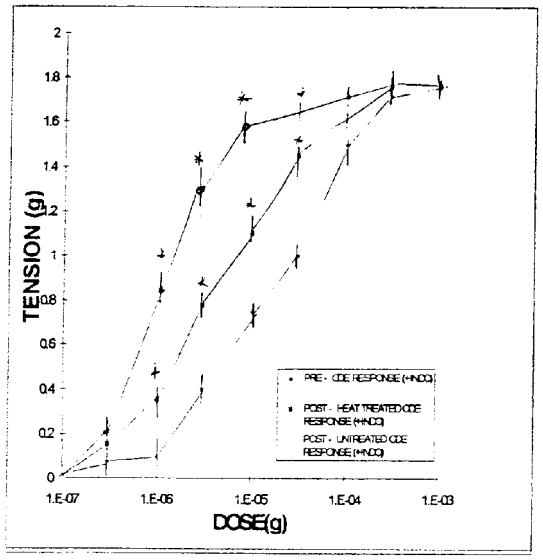

Figure 4. The effect of heat-treated and untreated CDE in the presence of indomethacin on tracheal reactivity to methacholine. *indicates $p<0.05$. n=6. Students t-test.

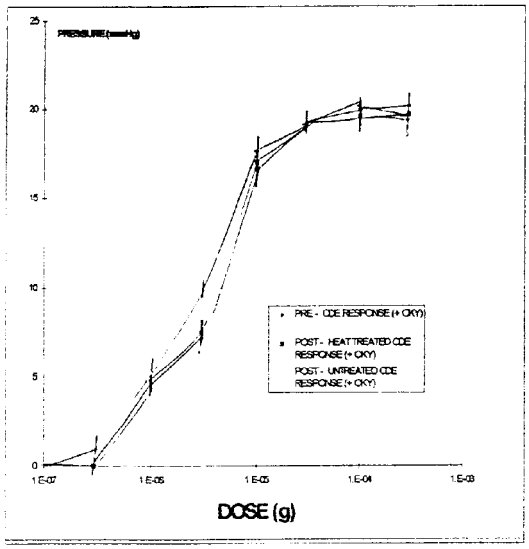

Figure 5. The effect of heat-treated and untreated CDE in the presence of OKY048 on pulmonary reactivity to methacholine. *indicates $p<0.05$. n=6. Students t-test.

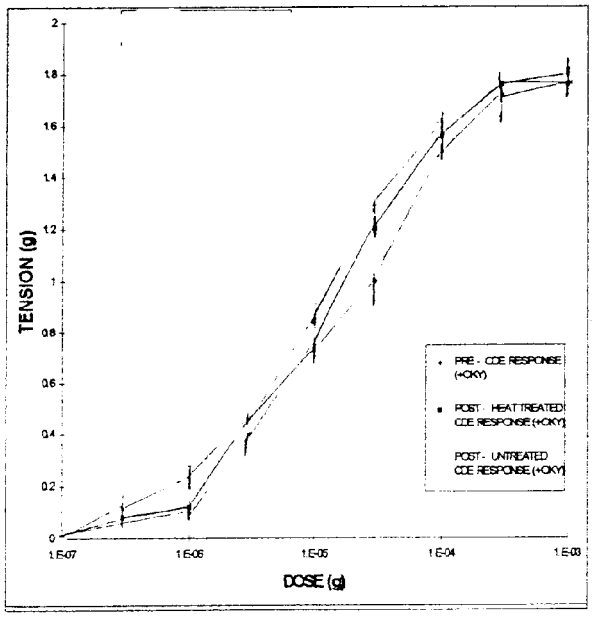

Figure 6. The effect of heat-treated and untreated CDE in the presence of OKY048 on tracheal reactivity to methacholine. *indicates $p<0.05$. n=6. Students t-test.

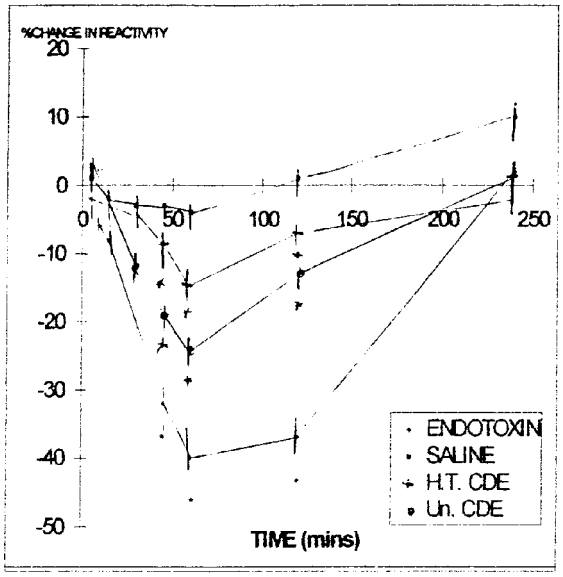

Figure 7. The effect of heat-treated and untreated CDE on *in vivo* pulmonary reactivity to methacholine. *indicates $p<0.05$. n=8. Students t-test.