

ENDOTOXIN AND  $\beta$ -(1,3)-GLUCAN LEVELS IN AUTOMOBILES: A PILOT STUDY

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**Abstract:** Exposure to bacterial endotoxin and fungal  $\beta$ -(1,3)-glucan in the indoor environment can induce respiratory symptoms. Automobiles are an exposure source of allergens but it is not known if, and how much exposure there is to endotoxin and fungal  $\beta$ -(1,3)-glucan. The objective of the study was to determine whether automobiles are a potential source of exposure to these microbial products. Dust was sampled from the passenger seats of 40 automobiles. Specific *Limulus* amoebocyte kinetic assays were used to measure endotoxin and  $\beta$ -(1,3)-glucan, respectively. Endotoxin and  $\beta$ -(1,3)-glucan was detected in all samples ranging from 19.9–247.0 EU/mg and 1.6–59.8  $\mu$ g/g, respectively. There were no significant differences in endotoxin levels between automobiles of smokers and non-smokers, but  $\beta$ -(1,3)-glucan levels were about two-fold higher in the automobiles of non-smokers. In conclusion, endotoxin and  $\beta$ -(1,3)-glucan exposure in automobiles at levels found in our study may be of importance for asthmatics.

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## INTRODUCTION

Asthmatic patients are exposed to a variety of insect and animal allergens and microbial products in the indoor environment. Exposure to allergens can provoke asthma symptoms in those sensitized and exposure to microbial products can both induce respiratory symptoms and augment responses to inhaled allergens [1, 3, 9, 10]. The majority of exposure studies have been performed in the domestic and work environments, where individuals can spend up to 90% of their time. Most of these studies have focussed on allergens from house dust mites, cockroaches, cats and dogs, and the microbial products, endotoxin and  $\beta$ -(1,3)-glucan from Gram-negative bacteria and fungi, respectively.

It is well known that exposure to house dust mite allergens can induce asthma symptoms in house dust mite

sensitized asthmatics and that there is a dose-response relation with asthma severity [9]. Exposure to bacterial endotoxin in asthmatics, as well as in normal subjects, induces respiratory symptoms [10] and interacts with house dust mite allergens in the provocation of airway inflammation [1].  $\beta$ -(1,3)-glucan is a pro-inflammatory compound found in fungal cell walls and can cause lung function decline after inhalation [3, 14]. Thus, it is of importance to establish levels of allergens and microbial products in the indoor environment for risk assessment and planning of intervention studies.

Less is known about allergens and microbial products in the indoor environment other than that from domestic dwellings and the work environment. Automobiles potentially could be a micro-environment where asthmatics are exposed to allergens and microbial products for significant periods of time. Previously it has been demonstrated in

Brazil that private automobiles are a significant source of cat and dog allergens but not house dust mite allergens [18], although another study found significant amounts of house dust mite allergens in some American automobiles [12]. To our knowledge, there have been no published studies of endotoxin or  $\beta$ -(1,3)-glucan exposure in automobiles. Therefore, the aim of our pilot study was to assess endotoxin and  $\beta$ -(1,3)-glucan levels in automobiles and some factors that may affect these levels.

## MATERIALS AND METHODS

**Recruitment and dust sampling.** Staff members from the Changhua Christian Hospital were prospectively invited to take part in the study. We collected information from the automobile owners about whether the seats had either been cleaned or vacuumed recently and whether the occupants smoked in the automobile while driving. The study was approved by the Changhua Christian Hospital's Ethics Committee and all participants gave written informed consent.

The study took place in spring (3 March–20 May 2009) with the outside temperature ranging from 20°C–28°C. Reservoir dust samples were collected from 40 automobiles by vacuuming the front passengers seat for 1 minute from the horizontal part of the seat, followed by 1 minute from the upright part of the seat using a Goldstar (LG Electronics, Seoul, Korea) vacuum cleaner (1,100 Watts) with a 25  $\mu$ m nylon mesh bag fitted into the vacuum cleaner's inlet hose as previously described [21]. Total dust weights for the 2 minute samples were recorded.

**Cigarette analysis.** Three different brands of cigarettes (Dunhill, 555, Marlboro) were analysed for  $\beta$ -(1,3)-glucan content as below.

**$\beta$ -(1,3)-glucan analysis.** For  $\beta$ (1,3)-glucan analysis 5 mg of dust or cigarette tobacco was extracted with 5.0 ml of 0.3N NaOH, shaken for 30 minutes at room temperature, centrifuged for 10 minutes at 4,000 g, and supernatants stored at –20°C before analysis in one analytical batch.  $\beta$ -(1,3)-glucan in the supernatants were estimated in a 1:1,000 dilution with a modified *Limulus* amoebocyte kinetic assay specific for  $\beta$ -(1,3)-glucan (GlucateLL™, Cape Cod Inc., Falmouth, MA, USA), as previously described [21].  $\beta$ -(1,3)-glucan results were recorded as  $\mu$ g/g dust.

**Endotoxin analysis.** For endotoxin analysis 200 mg of dust was extracted with 5.0 ml of pyrogen-free water containing 0.05% Tween-20, shaken for 30 minutes at room temperature, centrifuged for 10 minutes at 1,000 g, and supernatants stored at –20°C before analysis in one analytical batch. Endotoxin in the supernatants was estimated in a 1:500 dilution with the *Limulus* amoebocyte kinetic assay (Bio Whittaker, Walkersville MD, USA), as previously described [20]. Endotoxin results were recorded as endotoxin units per milligram of dust (EU/mg).

**Table 1.** Endotoxin and  $\beta$ -(1,3)-glucan levels from car seats.

	Endotoxin EU/mg	$\beta$ -(1,3)-glucan $\mu$ g/g
Geometric mean	76.3	5.8
95% CI	63.7–91.4	4.7–7.1
Range of values	19.9–247.0	1.6–59.8

**Statistical analysis.** As both endotoxin and  $\beta$ -(1,3)-glucan have log-normal distributions, data were log-transformed and expressed as geometric mean levels with 95% confidence intervals (95% CI). Data analysis was conducted using the programme "R", version 2.9.1 (R Foundation for Statistical Computing, Vienna, Austria). Correlations between car seat cleaning, and smoking in automobiles and the automobile seat endotoxin and  $\beta$ -(1,3)-glucan levels were calculated using the non-parametric Spearman rank-order correlation coefficient. Since the study size was relatively small, we did not conduct multivariate analyses. Statistical significance was set at the  $p=0.05$  level.

## RESULTS

Endotoxin and  $\beta$ -(1,3)-glucan was detected in all 40 dust samples. Table 1 shows the geometric mean levels (95% CI) and range of values of endotoxin and  $\beta$ -(1,3)-glucan from the automobile seats. Twenty of the automobile owners regularly smoked whilst driving. There were no significant differences in endotoxin levels between automobiles of smokers and non-smokers (results not shown). However,  $\beta$ -(1,3)-glucan levels in automobiles of non-smokers were about two-fold higher than in automobiles of smokers (geometric mean levels: 4.3  $\mu$ g/g vs 7.9  $\mu$ g/g, respectively;  $p=0.0001$ ). Excluding one outlier (59.8  $\mu$ g/g, from a non-smoker car) did not change the statistical significant difference ( $p=0.0035$ ).

There was no correlation between car interior cleaning practices and endotoxin or  $\beta$ -(1,3)-glucan levels, although not many car owners cleaned the interior of their automobiles. Eight car owners recorded that they had vacuumed their automobiles, but the most recent was one month prior to the study. Similarly, only four car owners recorded that they had wiped their car seats, again the most recent as one month prior.

$\beta$ -(1,3)-glucan content of three different brands of cigarettes, Dunhill, 555 and Marlboro, were 6.3  $\mu$ g, 7.4  $\mu$ g, and 12.8  $\mu$ g per gram of tobacco, respectively.

## DISCUSSION

Our study has shown that endotoxin and  $\beta$ -(1,3)-glucan are readily detectable from automobile seats. We were able to compare these results with domestic levels in Taiwan and New Zealand as all measurements were carried out in the same laboratory with identical methodology. A recent international study has shown large inter-laboratory differences in measured endotoxin levels [2]; therefore, caution

is required when comparing endotoxin results between different studies if methods are not identical.

The geometric mean level of endotoxin from the Taiwanese automobile seats (76.3 EU/mg) is about three quarters from that found in central Taiwanese mattresses (108.4 EU/mg) [21], but is about three-fold higher than found in New Zealand domestic carpeted living-room floors (22.7 EU/mg) [19]. We have previously demonstrated that clothing could also be a significant exposure source of endotoxin with a geometric mean level of 17.5 EU/mg [11].

The levels of endotoxin in the indoor environment of automobiles could be of importance to patients as Boehlecke *et al.* have demonstrated that house dust mite allergen and endotoxin, at levels commonly encountered in the indoor environment, act synergistically to enhance airway response in atopic asthmatics [1]. Michel *et al.* have also demonstrated that asthma severity of atopic asthmatics correlates better with reservoir indoor endotoxin levels than with house dust mite allergen levels [10]. However, a recent international study has found an inverse association between domestic endotoxin levels and asthma in children [4]. Due to differences in endotoxin detection methods, no lower threshold level of endotoxin causing respiratory symptoms has yet been established.

The geometric mean  $\beta$ -(1,3)-glucan from the Taiwanese automobile seats (5.8  $\mu\text{g/g}$ ) is about four to five-fold lower than we have previously found in central Taiwanese mattresses (25.2  $\mu\text{g/g}$ ) and about five to six-fold lower than in New Zealand domestic carpeted bedroom floors (32.4  $\mu\text{g/g}$ ) [5, 21]. As for endotoxin, no lower threshold levels causing respiratory symptoms for  $\beta$ -(1,3)-glucan have yet been established.

A surprising finding was that  $\beta$ -(1,3)-glucan levels were about two-fold higher in the automobiles of non-smokers than automobiles of smokers, even when excluding one high outlier from an automobile of a non-smoker. We hypothesised that automobiles of smokers would have higher  $\beta$ -(1,3)-glucan and endotoxin levels as tobacco is known to have significant amounts of endotoxin and the fungal compound ergosterol [8]. A previous study had shown that air conditioning systems in automobiles can be a significant source of mould contamination and that these mould concentrations decreased when running the air conditioning systems at maximum [7]. Thus it is possible that non-smokers were less likely to use the air conditioners in their automobiles than smokers. It may also be that smokers were more likely to keep their car windows open and that this may have resulted in lower reservoir  $\beta$ -(1,3)-glucan levels, although we found no differences in reservoir endotoxin levels. However, we did not collect information on whether the automobiles had air conditioning systems or if they were regularly used, nor on window opening preferences of drivers.

A new finding was that cigarette tobacco contains a significant amount of  $\beta$ -(1,3)-glucan which, to our knowledge, has not been previously recorded although it is not

surprising as tobacco is known to contain the fungal compound ergosterol [8]. We did not test cigarette tobacco for endotoxin as that is already known to contain significant amounts [17]. The combination of  $\beta$ -(1,3)-glucan and endotoxin in cigarette tobacco could be of health importance as it is known that exposure to  $\beta$ -(1,3)-glucan and tobacco smoke (containing endotoxin) synergistically increases inflammation in the lung [17].

As for endotoxin, levels of  $\beta$ -(1,3)-glucan in the indoor environment of automobiles could be of importance to patients as  $\beta$ -(1,3)-glucan inhalation has been associated with non-atopic respiratory symptoms [3, 14]. Although a review of  $\beta$ -(1,3)-glucan and respiratory health suggested some associations with airway inflammation and respiratory symptoms, larger and well designed observational studies are required to demonstrate the potential health aspects of indoor  $\beta$ -(1,3)-glucan exposure [3]. Whether  $\beta$ -(1,3)-glucan from automobile seats are significant in terms of human exposure depends on how and how much would be airborne, the length of time the driver or passengers are exposed and whether other car conditions, such as ventilation status.

A limitation of our study is the relatively small sample size. It is possible that endotoxin and  $\beta$ -(1,3)-glucan levels could be different in automobiles in other locations as studies have shown wide differences in endotoxin levels between countries [4], and we have previously shown differences in  $\beta$ -(1,3)-glucan levels in domestic dwellings between Taiwan and New Zealand [5, 21]. Furthermore, we only collected limited information and thus could have missed some significant associations of endotoxin and  $\beta$ -(1,3)-glucan exposure in automobiles, such as natural or mechanical ventilation. We also did not determine whether the automobile owners carried dogs as passengers. Dog ownership in homes has been positively associated with higher endotoxin levels in carpets in domestic dwellings [19].

Reservoir dust may not be the best matrix to assess environmental exposure. Airborne levels may be more relevant as, for instance, air levels of endotoxin are 4–63 times higher in homes of smokers than non-smokers, which could explain the prevalence of respiratory symptoms among smokers [15].

How endotoxin and  $\beta$ -(1,3)-glucan is dispersed in automobile interiors is, as yet, unknown. Most likely dispersal is from the outside environment where Gram-negative bacteria, as a source of endotoxin, and fungi, as a source of  $\beta$ -(1,3)-glucan, are ubiquitous. Possibly other contributions are dispersal from clothing and from spilt tobacco from cigarettes.

In conclusion, we have shown that automobiles are potentially a significant source of endotoxin and  $\beta$ -(1,3)-glucan exposure that could be of importance for asthmatics. This depends on the amount of time one spends in this environment and what levels in reservoir dust are important in provoking symptoms. Automobiles can now be

considered an indoor environmental source of endotoxin and  $\beta$ -(1,3)-glucan exposure, together with other indoor environments such as domestic dwellings [21], clothing [11, 16], schools [22], day-care centres [13], and even aeroplanes [6] with regard to the latter, endotoxin levels in automobiles were about half of those levels found from carpets in aeroplanes, although this needs to be interpreted with caution given the large inter-laboratory differences in measured endotoxin levels [2].

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