

Inhaled endotoxin and organic dust particulates have synergistic proinflammatory effects in equine heaves (organic dust-induced asthma)

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Summary

Background Equine heaves is a naturally occurring organic dust-induced asthma characterized by airway neutrophilia, mucus hypersecretion and obstructive lung dysfunction. However, the relative role of different dust components in disease severity remains unclear.

Objective This study investigated the relative contribution of inhaled endotoxin and organic dust particulates (mainly mould spores) in inducing heaves in heaves-susceptible horses.

Methods Control and heaves-susceptible horses received inhalation challenges with hay dust suspension (HDS) before and after lipopolysaccharide (LPS) depletion. Heaves-susceptible horses also received inhalation challenge with HDS particulates with and without the addition of LPS and were housed in two separate dusty environments during which mould and endotoxin exposure was measured. The airway inflammatory and functional response to each challenge was measured.

Results Depletion of endotoxin from HDS attenuated the airway neutrophilia and abrogated the airway dysfunction induced in heaves horses by inhaled HDS. The airway response was re-established by adding back LPS to the depleted HDS, confirming that the attenuation in airway response was due specifically to endotoxin depletion. Interestingly, the magnitude of alteration in airway response following endotoxin depletion and add-back was greater than that which could be attributed solely to endotoxin *per se*, indicating that the LPS activity was enhanced by the other dust components. Consistent with this possibility, washed particulates harvested from HDS enhanced the airway response to inhaled LPS in heaves horses. Heaves horses given two different hay/straw challenges had a significantly different severity of airway inflammation and dysfunction, despite airborne dust and endotoxin concentrations in the horses' breathing zones being similar.

Conclusion Although inhaled endotoxin appears not to be the only determinant of disease severity in heaves, it does contribute significantly to the induction of airway inflammation and dysfunction. This contribution is largely via the synergistic action of inhaled endotoxin and organic dust particulates, although other soluble dust components also contribute to a lesser degree.

Keywords endotoxin, heaves, mould spores, organic dust, particulates

Submitted 10 May 2002; revised 27 September 2002; accepted 13 December 2002

Introduction

Equine heaves resembles those types of human occupational asthma that occur in workers exposed to airborne organic dusts [1], being characterized by airway neutrophilia, mucus hypersecretion and airway obstruction [2]. The disease is induced by inhalation of airborne dust present in stables, and is reversible following use of low-dust feed and bedding, or turnout to pasture [2]. As with human organic dust-induced diseases [3–5], the relative importance of different dust components in inducing heaves is unknown [1, 6], and warrants further study [7]. While previous work suggested that heaves is a hypersensitivity response to inhaled moulds [6, 8–10], experimental aqueous mould extract inhalation has been shown to induce pulmonary neutrophilic inflammation and dysfunction,

consistent with, but less severe than, the natural disease [6]. This shortfall in response may reflect insufficient dose of mould and/or involvement of other dust components in the aetiopathogenesis of heaves [6]. Inhaled endotoxin is a likely candidate for involvement in heaves for a variety of reasons [11]. First, equine stables contain high concentrations of airborne endotoxin [12–15]. Second, the clinicopathological features of heaves resemble those of endotoxin-mediated lung disease in other species. Finally, short-term soluble lipopolysaccharide (LPS) inhalation induces many of the features of heaves in susceptible horses [11]. While the magnitude of soluble LPS exposure required to induce airway inflammation and dysfunction in short-term experimental inhalation challenges greatly exceeds that encountered in the stable [11], this potential discrepancy may reflect the limitations in short duration experimental LPS challenges when compared with longer duration natural LPS exposure [16]. Recently, a nebulized aqueous hay dust suspension (HDS)-induced disease model has been used successfully to reproduce the features of heaves in susceptible

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horses, to differentiate susceptible from control horses, and to demonstrate the importance of both soluble and particulate components in the induction of heaves [17–19]. This model was used in the present study, as it provides a method for investigating the relative importance of dust components, including endotoxin, in heaves. This paper describes three experiments performed to determine the importance of inhaled endotoxin in heaves. First, to establish whether endotoxin contributes to the airway response to inhaled HDS, the effect of LPS depletion and subsequent add-back was determined. Second, to determine whether the level of airborne endotoxin encountered in horse stables influenced the clinical severity of heaves, six heaves horses were housed in two different stable environments, and the severity of the induced airway inflammation and dysfunction was compared with the respirable and total airborne dust and endotoxin concentrations in these stables. Third, we determined whether co-challenge with dust particulates harvested from HDS would potentiate the airway response to inhaled LPS.

Materials and methods

Horses

Six healthy female control horses with no detectable respiratory tract disorders (median age 6 years, range 4–9 years; median weight 320 kg, range 316–356 kg) and seven horses (three geldings, four mares; aged 17 years, range 8–28 years; weight 434 kg, range 323–594 kg) with a history and clinical diagnosis of heaves were used. The disease status of all subjects was confirmed by previous mouldy hay/straw challenges of varying duration. These challenges induced three or more of the following: bronchoalveolar lavage fluid (BALF) neutrophilia ($>20\%$), increased volume of tracheal secretions, a reduced PaO_2 , a greater than twofold increase in maximum transpulmonary pressure and isovolumetric lung resistance, a greater than threefold increase in work of breathing, and a greater than 50% decrease in dynamic compliance in heaves horses. All of the above clinical and laboratory abnormalities reverted to normal when the heaves horses were moved to a low-dust environment. With the exception of the hay/straw challenges A and B (*vide infra*), all horses were kept in a low-dust environment throughout the duration of the study. The study was approved by the Home Office, and conducted under a Home Office project licence.

Inhalation challenges

Horses received inhalation challenges with saline (Vetivex, 0.9% w/v sodium chloride, Ivex Pharmaceuticals, Larne, UK) as a negative control challenge, and a previously described aqueous HDS [17] as a positive control for the heaves horses. To assess the importance of endotoxin in the response to HDS, six heaves horses and six control horses received endotoxin-depleted hay dust suspension (HDS-LPS), which was prepared by endotoxin adsorption onto polymixin-coated beads suspended in 50% glycerol. HDS was centrifuged at 1600 g for 15 min to yield pelleted dust particulates and supernatant. The supernatant, which contained 83% of the endotoxin activity present in HDS [19], was decanted and mixed with polymixin B-coated agarose beads (polymixin B-agarose, Sigma Aldrich Co, Poole, Dorset, UK) for 30 min. The mixture was further centrifuged at 1600 g

for 15 min to permit removal of the polymixin-coated agarose beads, and the resultant solution used to reconstitute the dust particulates to yield HDS-LPS. As it was not possible to pellet the glycerol along with the polymixin-coated beads, the final volume of HDS-LPS was greater than the original volume of HDS (40% increase in volume). The endotoxin concentration of HDS-LPS (21 850 EU/mL) was 42% of that for HDS (51 840 EU/mL), as determined using an endotoxin-specific assay (Endospecy, Seikagaku Co., Tokyo, Japan), performed according to Thorn [20]. To ensure that any alteration in response following endotoxin depletion was due solely to depletion of endotoxin, the same six heaves horses also received inhalation challenge with HDS-LPS + LPS which was prepared by adding back soluble LPS (*Salmonella typhimurium* Ra60, donated by Professor I. Poxton, Medical Microbiology, University of Edinburgh) from a stock solution of 21 336 000 EU/mL, at a quantity equivalent to that which had been removed during the depletion. Additionally, to determine whether co-inhalation with dust particulates enhanced the response to inhaled endotoxin, all seven heaves horses received a combined inhalation challenge with LPS (48 000 EU) and washed particulates (WP), which were prepared from HDS by repeated centrifugation and washing as previously described [19]. This challenge was referred to as WP + LPS.

To minimize potential carry-over effects from preceding challenges, challenges were conducted a minimum of 14 days apart, and all horses were shown to have normal clinical findings prior to each challenge. In addition, six heaves horses received a repeat challenge with WP + LPS after completion of all other challenges, both to assess potential carry over effects, and to determine the repeatability of this challenge.

Inhalation challenge was performed as previously described [11]. To facilitate nebulization, horses were sedated with 20 µg/kg romifidine (Sedivet, Boehringer Ingelheim Ltd, Bracknell, Berkshire, UK) and 10 µg/kg butorphanol (Torbugesic, Fort Dodge Animal Health, Southampton, UK), intravenously. A compressor (Parimaster, PARI Medical Ltd, West Byfleet, Surrey, UK), with a calibrated output of 7 L/min, connected to a nebulizer cup (Sidestream, Medic-Aid Ltd, Bognor Regis, West Sussex, UK) containing 2 mL of challenge substance, was used to produce an aerosol which comprised 80% respirable particles $<5\text{ }\mu\text{m}$ (as reported by the manufacturer at the flow rate used). The aerosol passed via the inspiratory arm of a 'Y-piece' delivery device into an airtight facemask. During expiration, expired breath passed via a one-way valve in the expiratory arm of the delivery device, and was vented to the external environment. One millilitre of solution/suspension was delivered to the facemask for each challenge, with exception of the HDS-LPS and HDS-LPS + LPS challenges, when 1.4 mL suspension was delivered to correct for the dilution effects of glycerol. Consequently, polymixin treatment of HDS reduced the final endotoxin exposure at the facemask from 51 840 EU for HDS to 30 590 EU for HDS-LPS (21 850 EU/mL $\times$ 1.4 mL), equating to a 41% reduction. Any further dilution of HDS-LPS following add-back of LPS to produce HDS-LPS + LPS was considered negligible.

Hay/straw challenges

For the two hay/straw challenges (A and B), six heaves horses were housed for 5 h in two small (3.7 $\times$ 3.7 m), poorly ventilated

stables with the doors and air vents closed, bedded on deep litter straw, and fed a mixture of good quality and dusty hay. Only the hay used in stable B had visible mould growth. This hay/straw challenge has been shown to induce heaves only in susceptible horses [6]. Throughout the 5-h duration of these challenges, total and respirable stable dust was collected from the breathing zones of all horses using personal samplers (AFC124 High Flow Personal Sampler, Casella Ltd, Kempston, UK), as previously described [15]. Dust samples were prepared for endotoxin analysis as described previously [15], but were not filtered after elution of soluble components from the filter membrane. The endotoxin content of the dust filter eluent was determined using an endotoxin-specific *Limulus* amoebocyte lysate assay (Endospecy, Seikagaku Co) as described by Thorn [20]. As a measure of airborne mould exposure, the β -D-glucan content of the dust filter eluent was also determined measured using a glucan-specific *Limulus* amoebocyte lysate assay (Gluspecy, Seikagaku Co) as described by Thorn [20], with the exception of the addition of sodium hydroxide to the eluent.

The median time interval between the two separate hay/straw challenges was 33 days (range 14–44 days). All horses were shown to have normal baseline BALF cytology ($< 5\%$ neutrophils) prior to entering stable B. The baseline BALF sample was obtained from the left ventral lung segment, and the median time interval between collection of this sample and entry into stable B was 11 days (range 7–27 days).

Assessment of response to challenges

The response to challenges was assessed using clinical scoring, lung mechanics, airway reactivity and BALF cytology as previously described [11, 18, 21], except for repeat WP + LPS challenge which was assessed using BALF cytology only (Fig. 1). To ensure subject compliance, all horses were sedated, as described above, immediately prior to measuring airway reactivity. All BALF samples obtained at 6 h following challenge were obtained from the right accessory lung lobe. Arterial blood gas analyses was also used to assess the response to hay/straw challenges.

Statistical analyses

The effect of each inhalation challenge was determined by performing within-group analyses. Where pre-challenge measurements were made at $t = -30$ min (arterial blood gas analyses and lung mechanics) the post-challenge values were expressed as percentage of baseline value, except for clinical scores where actual values were used. As the vehicle for all nebulized inhalation challenges was saline, the effect of these challenges was assessed by pairing and subtracting post-challenge and post-saline data. Where no pre-challenge data were collected, comparisons were made with saline challenge data. A Friedman test

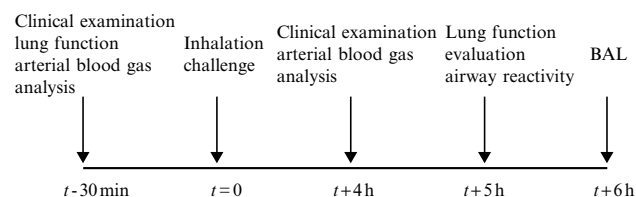


Fig. 1. Study design. For the 5-h duration hay/straw challenges, the horses entered the stable at $t = 0$. BAL, bronchoalveolar lavage.

was performed on sets of paired data, and when significant, a Wilcoxon rank sum test was performed. Significance was assumed if $P < 0.05$. The two separate WP + LPS inhalation challenges were compared using a Wilcoxon rank sum test, and as an indication of repeatability, the differences in paired values were plotted against their mean as described by Bland and Altman [22]. Good repeatability was assumed if the calculated differences in paired values fell within 2SD of the mean of the differences [23]. Changes from baseline and comparison of medians between challenges are expressed as increase/decrease or difference in median values, with 95% confidence interval (CI) for the difference in median, calculated for non-parametric data as described by Campbell and Gardner [24].

Results

Response to inhalation challenges with HDS, HDS-LPS and HDS-LPS + LPS

Heaves horses had significantly ($P < 0.05$) increased BALF neutrophil ratios and counts after HDS (difference in median count $2.25 \times 10^5/\text{mL}$, 95% CI 0.76–3.74), HDS-LPS (difference in median count $0.92 \times 10^5/\text{mL}$, 95% CI 0.29–2.40) and HDS-LPS + LPS (difference in median count $1.68 \times 10^5/\text{mL}$, 95% CI 0.53–4.21), when compared with saline (Table 1, Fig. 2). While BALF neutrophil counts and ratios after HDS and HDS-LPS + LPS did not differ, they were significantly ($P < 0.05$) higher than those after HDS-LPS (differences in median counts $1.27 \times 10^5/\text{mL}$, 95% CI 0.13–0.62 and $0.82 \times 10^5/\text{mL}$, 95% CI 0.07–2.65, respectively; Table 1, Fig. 2). In heaves horses, absolute BALF macrophage numbers after HDS-LPS + LPS were significantly ($P < 0.05$) lower than those after saline (differences in median $0.85 \times 10^5/\text{mL}$, 95% CI 0.14–1.58) and HDS-LPS (difference in median $0.40 \times 10^5/\text{mL}$, 95% CI 0.01–1.10), but not significantly different from HDS. In heaves horses, absolute BALF mast cell counts were significantly ($P < 0.05$) reduced by HDS (difference in median $0.07 \times 10^5/\text{mL}$, 95% CI 0.03–0.14) and HDS-LPS (difference in median $0.08 \times 10^5/\text{mL}$, 95% CI 0.01–0.15), compared with saline. In heaves horses, absolute BALF lymphocyte counts

Table 1. BALF cell counts ($\times 10^5/\text{mL}$) in heaves horses ($n = 6$) at 6 h following inhalation challenge with saline, HDS, HDS-LPS and HDS-LPS + LPS (median and range)

	Saline	HDS	HDS-LPS	HDS-LPS + LPS
Total cell count	4.50 (3.20–5.60)	4.10 (3.70–7.00)	3.75 (3.00–6.20)	4.05 (3.40–6.00)
Neutrophils	0.07†‡ (0.03–0.20)	2.39*‡ (0.80–3.81)	0.54*† (0.44–2.48)	1.87*‡ (0.56–4.28)
Macrophages	1.77 (1.24–2.96)	1.17 (0.41–1.75)	1.30 (0.67–2.16)	1.07*‡ (0.66–1.38)
Mast cells	0.14†‡ (0.09–0.19)	0.06* (0.02–0.11)	0.04* (0.01–0.14)	0.07 (0.00–0.18)
Lymphocytes	2.22 (1.54–3.33)	1.41 (0.61–1.86)	1.37 (0.90–2.43)	1.07* (0.61–2.35)
Eosinophils	0.01 (0.00–0.01)	0.01 (0.00–0.13)	0.00 (0.00–0.06)	0.01 (0.00–0.04)

*Statistically different from saline ($P < 0.05$); †statistically different from HDS ($P < 0.05$); ‡statistically different from HDS-LPS ($P < 0.05$).

were reduced by HDS-LPS + LPS (difference in median $0.97 \times 10^5/\text{mL}$, 95% CI 0.58–1.30), and non-significantly ($P = 0.059$) reduced by HDS (difference in median $0.91 \times 10^5/\text{mL}$, 90% CI 0.34–1.45), compared with saline.

After HDS challenge, control horses had a slight yet significant ($P < 0.05$) increase in BALF neutrophil count and ratio at 6 h, when compared with saline (difference in median count $0.26 \times 10^5/\text{mL}$, 95% CI 0.06–0.49) and an increase, which approached significance ($P = 0.059$), when compared with HDS-LPS (difference in median count $0.23 \times 10^5/\text{mL}$, 90% CI 0.03–0.49; Fig. 2). The BALF neutrophil count and ratio of controls following HDS-LPS and saline challenges did not differ significantly (Fig. 2).

In heaves horses, only HDS and HDS-LPS + LPS induced pulmonary dysfunction. HDS induced a significant ($P < 0.05$) increase in expiratory resistive work of breathing (Wb_{Eres}) (increase in median 66%, 95% CI 8–157), and a non-significant ($P = 0.059$) increase in $\text{R}_{\text{LE75\%}}$ (Fig. 3). HDS-LPS + LPS induced a significant ($P < 0.05$) increase in both $\text{R}_{\text{LE25\%}}$ (increase in median 142%, 95% CI 32–363) and $\text{R}_{\text{LE75\%}}$ (increase in median 103%, 95% CI 15–192; Fig. 3). The challenges did not

alter airway reactivity or clinical score in either group, and did not alter lung function in controls.

Response to inhalation challenge with WP + LPS

BALF neutrophil counts and ratios following inhalation challenge with WP + LPS are presented in Table 2 and Fig. 4. Previous data obtained from the same seven heaves horses following inhalation challenges with 48 000 EU (20 μg) LPS, WP, SUP (HDS supernatant) and WP + SUP is also presented for comparison [11, 19]. WP + LPS induced an airway neutrophilia which was significantly ($P < 0.05$) greater than that for saline (increase in median count $0.89 \times 10^5/\text{mL}$, 95% CI 0.42–1.45) and only slightly, yet significantly ($P < 0.05$), lower than that for HDS (Table 2, Fig. 4). The responses of other BALF cells to HDS and WP + LPS did not differ significantly. WP + LPS induced airway dysfunction as evidenced by a significant increase in $\text{R}_{\text{LE25\%}}$ (increase in median 103%, 95% CI 30–191) compared with saline (Fig. 5), but did not alter airway reactivity or clinical score.

There was no significant difference in the number of any BALF cell type between the first and second WP + LPS

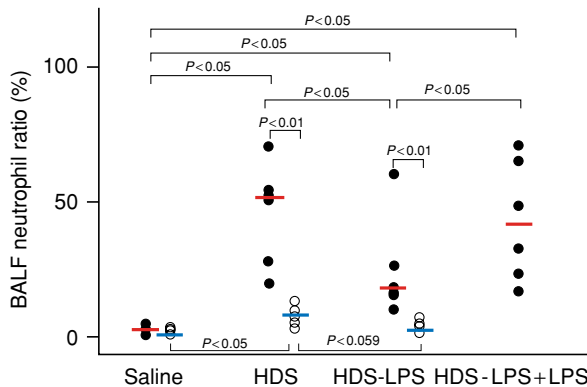


Fig. 2. BALF neutrophil ratio (%) in heaves ($n = 6$) horses at 6 h following inhalation challenge with saline, HDS, HDS-LPS and HDS-LPS + LPS, and in control ($n = 6$) horses 6 h following inhalation challenge with saline, HDS and HDS-LPS. ●, heaves group; ○, control group; horizontal lines, median value for group.

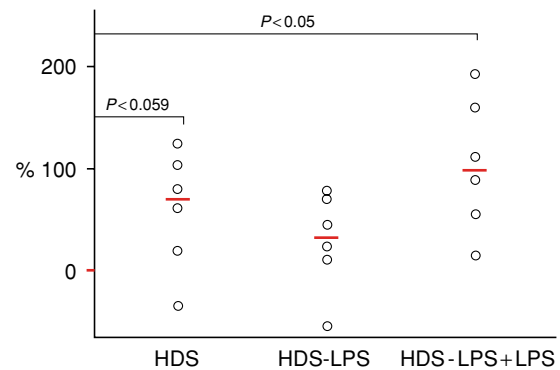


Fig. 3. Percentage of baseline $\text{R}_{\text{LE75\%}}$ in heaves horses ($n = 6$) at 5 h following inhalation challenge with HDS, HDS-LPS and HDS-LPS + LPS minus percentage of baseline $\text{R}_{\text{LE75\%}}$, at 5 h following inhalation challenge with saline (= 0%). Horizontal lines, median value.

Table 2. BALF cell counts ($\times 10^5/\text{mL}$) in seven heaves horses at 6 h following inhalation challenge with saline, WP + LPS and HDS (median and range)

	Saline	LPS*	WP*	SUP*	WP + LPS	WP + SUP*	HDS
Total cell count	3.80 (1.30–5.60)	4.60** (2.90–7.40)	5.50†** (2.00–9.10)	7.30†** (3.40–9.40)	3.80‡§¶†† (2.30–4.20)	5.40** (2.30–10.2)	4.10†† (2.10–7.00)
Neutrophils	0.06‡§¶†† (0.03–0.20)	0.28†** (0.18–0.53)	0.19†**†† (0.10–0.65)	0.53††† (0.28–1.64)	0.71†‡§ (0.40–1.62)	1.36†§¶ (0.43–4.55)	2.17†§¶††† (0.54–3.81)
Macrophages	1.58 (0.34–2.96)	1.68** (0.70–2.57)	1.99** (0.71–3.92)	1.98** (0.55–4.21)	0.89‡§¶†† (0.36–1.51)	1.46** (0.38–4.81)	1.16§¶†† (0.41–1.75)
Mast cells	0.13 (0.09–0.19)	0.07 (0.06–0.30)	0.14** (0.09–0.23)	0.13 (0.02–0.23)	0.09§ (0.00–0.14)	0.08 (0.02–0.24)	0.07§ (0.02–0.14)
Lymphocytes	2.09 (0.80–3.33)	2.45** (1.88–4.83)	2.21† (0.95–5.20)	2.94** (1.63–5.61)	1.38‡§¶†† (0.81–2.08)	2.33** (1.09–2.83)	1.19‡§¶††† (0.61–1.86)
Eosinophils	0.01¶ (0.00–0.01)	0.00§¶ (0.00–0.01)	0.07‡ (0.00–1.54)	0.07†‡** (0.01–0.33)	0.01¶ (0.00–0.01)	0.01 (0.00–0.20)	0.01 (0.00–0.13)

BALF cellular responses in the same horses 6 h following challenge with LPS, WP, SUP and WP + SUP are included for comparison (*published data [19]). †Statistically different from saline ($P < 0.05$); ‡statistically different from LPS ($P < 0.05$); §statistically different from WP ($P < 0.05$); ¶statistically different from SUP ($P < 0.05$); **statistically different from WP + LPS ($P < 0.05$); ††statistically different from WP + SUP ($P < 0.05$).

challenges, except for a significant ($P < 0.05$) elevation in macrophage count after the second challenge. As all of the six calculated differences in paired values fell within 2SD of the mean of the differences for all BALF cell types, and the mean of the differences approximated zero, except for macrophage numbers, repeatability was considered good [22].

Hay/straw challenges A and B

There was no significant difference in airborne dust and endotoxin exposure during hay/straw challenges A and B (Table 3), although the respirable dust endotoxin concentration was

non-significantly ($P = 0.059$) higher during challenge A (Table 3). Conversely, and in agreement with the visible mould contamination of the hay, the respirable dust β -D-glucan concentration was non-significantly ($P = 0.059$) higher during challenge B (Table 3). Challenge B induced a significantly ($P < 0.05$) higher BALF neutrophil count and ratio than challenge A (Table 3). Challenge B induced a significant ($P < 0.05$) arterial hypoxaemia at 90 min (median decrease in PaO_2 4%, 95% CI 1–19), with a similar but non-significant ($P = 0.059$) reduction at 4 h. Hay/straw challenges did not alter pulmonary mechanics, airway reactivity or clinical score.

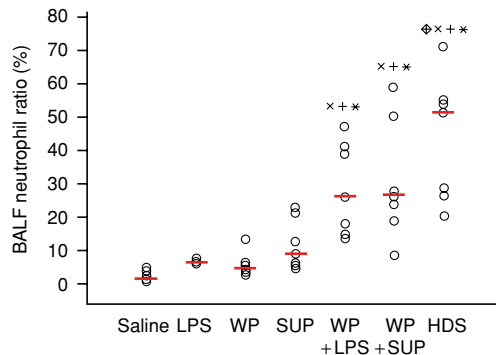


Fig. 4. BALF neutrophil ratio (%) in heaves ($n = 7$) horses at 6 h following inhalation challenge with saline, 20 μg LPS, and WP + LPS. Previously published data in the same horses following inhalation challenge with WP, SUP (HDS supernatant), WP + SUP and HDS is included for comparison. Horizontal lines, median value for group; all challenges, significantly different from saline ($P < 0.05$); *, significantly different from 20 μg LPS; +, significantly different from WP; x, significantly different from SUP; ϕ , significantly different from WP + LPS.

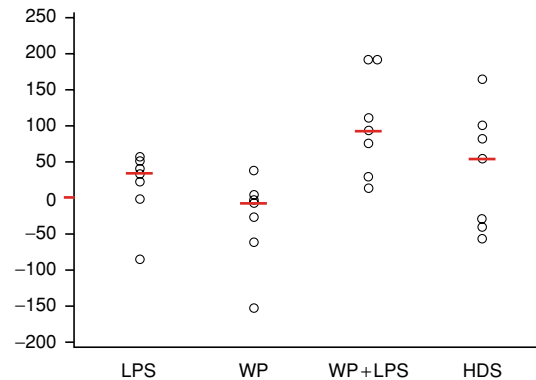


Fig. 5. Percentage of baseline $R_{LE25\%}$ in heaves horses ($n = 7$) at 5 h following inhalation challenge with 20 μg LPS, WP, WP + 20 μg LPS and HDS minus percentage of baseline $R_{LE75\%}$, at 5 h following inhalation challenge with saline (= 0%). Horizontal lines, median value.

Table 3. Total and respirable airborne dust, and endotoxin and β -D-glucan concentrations and content of total and respirable dust collected during hay straw challenge A and B, and the resultant BALF neutrophil ratio and count at 6 h (median and range)

	Hay/straw challenge A	Hay/straw challenge B
Total dust concentration (mg/m^3)	1.2 (0.7–1.7)	1.5 (0.5–1.9)
Respirable dust concentration (mg/m^3)	0.1 (0.1–0.6)	0.2 (0.1–0.3)
Respirable β -D-glucan concentration (ng/m^3)	3.5 (2.3–6.2)	8.5 $\dagger$ (3.2–16.8)
Respirable dust β -D-glucan content (ng/mg)	24.6 (10.3–36.0)	42.6 (15.8–168.4)
Total endotoxin concentration (ng/m^3)	156 (36–412)	138 (87–427)
Respirable endotoxin concentration (ng/m^3)	6.9 (2.9–38.7)	3.6 $\dagger$ (1.8–4.3)
Total dust endotoxin content (ng/mg)	83 (30–145)	67 (37–150)
Respirable dust endotoxin content (ng/mg)	32.0 (7.8–71.3)	9.0 (5.3–25.8)
Absolute BALF neutrophil count ($\times 10^6/\text{mL}$)	0.45 (0.34–0.70)	2.05* (0.74–9.83)
BALF neutrophil ratio (%)	9.9 (4.5–16.0)	36.0* (21.0–60.7)

*Significantly different from hay/straw challenge A ($P < 0.05$); $\dagger$ difference from hay/straw challenge A, approaching significance ($P = 0.059$).

Discussion

This study highlights the contributory role of inhaled LPS to airway inflammation and dysfunction in equine heaves. Reduction of the LPS content of an aqueous HDS significantly attenuated the airway neutrophilia and dysfunction induced by inhalation challenge in heaves horses, and non-significantly reduced the airway neutrophilia in controls. The airway inflammatory and functional response was almost completely re-established in heaves horses after adding back endotoxin to the depleted HDS, confirming that the attenuation was due almost entirely to reduction in endotoxin. It could be argued that any slight reduction in potency of HDS-LPS + LPS compared with HDS might be attributed to the effect of glycerol contamination or a reduction in other negatively charged soluble components of HDS following treatment with the polycation, polymixin. The reduction in airway inflammatory response following endotoxin depletion was, however, less marked than that reported following LPS depletion of corn dust extract in a mouse model of human organic dust-induced disease [25]. This may be explained by the removal of a lower proportion of LPS (41%) in the present study compared with the previous study (89%). Alternatively, other proinflammatory agents, such as dust particulates, which were present in HDS-LPS but not in the soluble corn dust extract used by Jagielo and colleagues [25], may have contributed to the airway response in the present study. In the present study, HDS containing both soluble and particulate dust components was used, as particulates contribute to the airway response to HDS [19]. While endotoxin could only be removed from the HDS supernatant and not from the particulates, this was not considered to be problematic as the supernatant contained 83% of the endotoxin activity of HDS [19], although the particulate endotoxin content may have been underestimated using the *Limulus* amoebocyte lysate assay [26].

Horses received an exposure to 30 590 EU endotoxin during the HDS-LPS challenge, compared with 51 840 EU for the HDS and HDS-LPS + LPS challenges. Interestingly, in the endotoxin depletion and add-back experiments, the magnitude of the airway response was altered to a greater extent than could be solely attributed to the direct action of 21 250 EU LPS. Indeed, previous studies indicate that inhalation challenge with 48 000 EU soluble LPS in the same heaves horses induced only mild airway neutrophilia ($0.28 \times 10^5/\text{mL}$; 0.18–0.53), without airway dysfunction [11]. This suggests that other components of HDS enhance the activity of the LPS, resulting in a more severe airway response than would have resulted from inhalation of LPS alone. Consistent with this hypothesis, addition of 48 000 EU soluble LPS enhanced the airway inflammatory response to washed particulates (predominantly washed fungal spores) harvested from HDS, to a greater extent than could be attributed to the effects of 48 000 EU LPS *per se*. This is in agreement with previous *in vivo* and *in vitro* studies which demonstrate that particulates enhance the response to proinflammatory agents such as LPS, and conversely that the airway response to inhaled particulates is enhanced by LPS priming [27–30].

Synergy between LPS and particulates in the current study may be due to binding of LPS to the organic particulates resulting in a more focal and concentrated LPS challenge, in comparison to the likely more diffuse deposition of an inhaled

solution [16]. Alternatively, while challenges with WP alone induce only a mild airway neutrophilia ($0.19 \times 10^5/\text{mL}$; 0.10–0.65) [19], they may prime target cells thereby enhancing LPS responsiveness. In this respect, both *in vitro* and *in vivo* priming of guinea pig alveolar macrophages has been reported following fungal antigen and glucan challenge [3, 31], both reported components of HDS [17, 19]. Lastly, in light of the results of previous inhalation studies that have proposed a hypersensitivity response in heaves, it is possible that the mould spores within the washed particulates represented a source of allergen. Consequently, mould spore inhalation may have resulted in an allergen-associated increase in LPS binding protein and soluble CD14 receptors in the airways, as reported in humans, thus magnifying the response to LPS [32, 33]. This synergy may in part explain why the magnitude of soluble LPS exposure necessary to induce airway inflammation and dysfunction in heaves horses is markedly greater than that to which horses are exposed naturally [11].

While this study has confirmed a role for endotoxin in the aetiopathogenesis of heaves, other findings indicate that additional dust components are also involved. First, heaves horses given hay/straw challenges A and B developed significant airway inflammation and dysfunction only after challenge B, despite the airborne dust and endotoxin exposure being similar for both challenges. This indicates that inhaled endotoxin is not the major determinant of the severity of heaves. The greater severity of airway response in challenge B may have resulted from inhalation of moulds from the visibly mouldy hay. Consistent with this possibility, previous equine studies have shown that inhalation of aqueous mould extracts results in airway inflammation and dysfunction resembling heaves [6, 8, 34]. Despite the visible mould contamination of the hay used in stable B, measurement of β -D-glucan in the respirable dust during both challenges permitted a more objective assessment of mould exposure [35]. Although the airborne β -D-glucan concentration was non-significantly greater in stable B, a clearer difference between the two environments may have been more evident had the filter eluents been treated with sodium hydroxide prior to analysis, a process reported to unwind the helical structure of the mould cell wall component [20]. Despite the normal baseline BALF cytology prior to challenge B, it could be argued that the pulmonary inflammatory response to this challenge may in part be due to a 'priming' effect of challenge A, particularly as the challenges were not randomized. However, previous work in the same group of horses has shown that using time intervals between challenges similar to those reported in this study, the repeatability of the pulmonary inflammatory response to repeated single doses of either HDS or LPS is good [11, 18].

Second, while heaves horses have a lower no-response threshold to inhaled LPS than controls, at the LPS exposures required to reproduce the airway inflammatory response found in the natural disease, there was no significant difference in airway response between the two groups [11]. In contrast, the pulmonary inflammatory response of control horses to HDS [18] and to hay/straw challenge [6] is markedly less than that of heaves horses.

Although the response to WP + LPS was greater than that to both WP and LPS alone, it was significantly less than that to non-fractionated HDS. This may have resulted from an enhancement of the airway response to LPS by other soluble

HDS components such as proteases [17, 19]. In agreement with this possibility, comparison with previous data demonstrated that challenge with WP + LPS resulted in a median BALF neutrophil count which appeared lower than that following challenge with WP resuspended in HDS supernatant (1.36×10^5 /mL; 0.43–4.55) [19]. However, the difference in BALF neutrophil counts between these challenges was not statistically significant. Indeed the neutrophilic response following these two challenges would have been almost identical had BALF neutrophil ratios (WP + LPS – 25.3%; 13.0–46.7; WP resuspended in HDS supernatant – 25.7; 7.9–58.3 [19]) been used as a measure of airway inflammation instead of BALF neutrophil counts. Therefore, despite the probable combined effects of LPS and other soluble HDS components, it is likely that the combination of LPS and particulates play a more important role in heaves.

It is possible that the reduction in BALF macrophage numbers following HDS-LPS + LPS challenge could be attributed largely to the effects of LPS. The finding that inhalation challenge with the same batch of LPS to that used in the HDS-LPS + LPS challenges, in the same group of horses also resulted in a non-significant reduction in BALF macrophage numbers [11] supports this explanation. However, this was only detected following extremely high LPS exposures (4 800 000 EU) which supports the role of other inhalants present within HDS magnifying the response to LPS in the heaves group. Additionally, this is supported by the finding that control horses also developed a significant reduction in BALF macrophage numbers following LPS inhalation [11], yet no reduction was detected following HDS inhalation in this group. A reduction in macrophage numbers in sputum has been reported following LPS inhalation in humans where LPS-induced macrophage apoptosis was proposed as a potential cause [36]. Additionally, reduced macrophage numbers have also been reported in heaves horses following mouldy hay exposure, a finding attributed to macrophage migration from the lung following clearance of apoptotic neutrophils [21]. Although a reduction in BALF mast cell numbers has been reported following LPS inhalation in horses [11], it is unlikely that the reduction detected following HDS inhalation could be attributed entirely to the LPS content. First, a similar reduction was also observed following HDS-LPS inhalation, and second, HDS-LPS + LPS failed to result in a significant reduction in BALF mast cell numbers, although in the latter instance a reduction was observed in five out of the six heaves horses. Reduced BALF mast cell numbers have been reported following mouldy hay challenge in heaves horses, whereby it was hypothesized that the reduction may reflect cell degranulation, with a failure to identify degranulated mast cell on cytospin preparations stained using Leishmans' stain [6]. Additionally, five of the six heaves horses had lower BALF mast cell numbers following housing in stable B, compared with stable A (data not shown), and fungal extract inhalation in the same group of horses also resulted in a reduction in BALF mast cell numbers (data not shown). Therefore it would appear that the mould content of HDS is more likely to contribute to this finding.

Despite the significant increase in pulmonary resistance measured following inhalation challenge with HDS, HDS + LPS and WP + LPS, it should be noted that this dysfunction was not of sufficient severity to be clinically detectable. This is likely to reflect the relative insensitivity of pulmonary mechanics testing in the horse when measured during tidal

breathing. Additionally, the absence of any significant alteration in airway reactivity following any of the challenges may result from insufficient challenge exposure; however, it may also reflect an attenuation of the methacholine-induced bronchoconstriction by the bronchodilatory effects of the α_2 -agonist drug [37] used to sedate horses for this procedure.

The endotoxin activity removed from HDS by polymyxin adsorption likely comprised a variety of LPS types, in contrast to the *S. typhimurium* Ra60 mutant used in the LPS challenge and add-back experiments. This Ra chemotype LPS (i.e. complete core oligosaccharide plus lipid A) was chosen as it is not possible to produce a pure endotoxin mix which is representative of those types of LPS encountered in equine stables. Further, the complete core oligosaccharide plus lipid A is responsible for a major part of the biological activity of LPS, and is likely present in high concentrations in HDS as this structure is shared by many Enterobacteriaceae (I. R. Poxton, personal communication).

In conclusion, this study has demonstrated that inhaled endotoxin contributes markedly to the airway inflammatory and functional response in heaves. In addition, the response to inhaled endotoxin is enhanced when copresented to the lung with other organic dust components, most notably dust particulates. This work has important implications for organic dust-induced disease in all species as it highlights the importance of quantifying both endotoxin and dust particulate exposure, and not considering each component in isolation.

Acknowledgements

This study was funded by the Horserace Betting Levy Board. The authors acknowledge the Wellcome Trust for the provision of equipment and facilities used in this study. They also gratefully acknowledge Catherine Lambert and Kelly Cole for technical assistance.

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