Supplementary Item 1: Methodological details.

Methods

Animals

The horses studied belonged to the research herd of Equine Asthma Research Laboratory of the Université de Montréal. Horses were housed in individual stalls inside a barn and allowed to exercise outside in a paddock for a fixed number of hours per day. They were bedded on shavings and/or hay and fed hay supplemented with a pelleted grain or whole feed ration. Water was available ad libitum. Horses had a history of lower airway obstruction following hay exposure (defined as R_L > 1 $cmH_2O/L/sec$). Exclusion criteria included treatment with corticosteroids, non-steroidal anti-inflammatory drugs, β-agonists, or other respiratory drugs within 3 weeks prior to enrollment into the study; a history of previous laminitis; pregnancy; the inability to fit the nostril adapter of the inhalative device into the left nostril due to anatomical or functional reasons; and the inability of the horse to tolerate treatment with the new inhalative device.

Experimental induction of personalized controlled equine asthma exacerbations

Before the beginning of each study, horses were tested for a variable period ranging from 2 to 4 weeks in order to find combinations of hay quantity and administration frequency per day that induced moderate-to-severe obstruction in each animal (corresponding to a clinical score of 5-6/8 based on the score proposed by Robinson and colleagues [1]). Horses were then exposed to a personalised hay challenge that remained unchanged for the duration of the study. This study design has previously been used for similar studies in asthmatic horses [2,3] as it permits to control for external sources of bias.

Treatments

Placebo inhalation started at day 0 PM of the relevant week (placebo week in study I and II, and treatment weeks in study III) and lasted for one week in studies I and II, and for 2 weeks in study III. Four actuations of placebo were administered twice daily in study I, 5 in study II, and 8 in study III.

Clinical assessment

During conditioning, complete physical examination and upper respiratory tract endoscopy were performed to exclude the presence of any sign of systemic/respiratory disease and any cause of upper airway obstruction. Respiratory obstruction and lameness were assessed using validated scores [1,4] as described below.

In study I, respiratory obstruction and lameness were assessed daily using previously published scores [1,4]. Briefly, the 8-point breathing effort score results from the assessment of nasal flaring and abdominal effort during breathing (1: normal; 4: severe). Due to the inability of the 8-point breathing effort score employed in study I to reflect the significant differences observed in lung function, a different 23-point weighted clinical score of the respiratory system was performed in studies II and III once a week (day 0 of placebo week and/or days 0, 7, 14, 21 of each cycle of treatment), always in the morning, prior to lung function measurement by an operator blinded to treatment groups. This score is based on the system proposed by Hoffman and colleagues [5] and subsequently modified and employed for the assessment of airway obstruction in asthmatic horses [6]. With the weighted clinical score, bronchial tones, crackles and wheezes are assessed when the horse is spontaneously breathing.

The following scheme present the parameters assessed with the weighted clinical score and their scores:

Variable	**Descriptor**	**Score**
Respiratory rate (breaths/min)	<16	0
	16-20	1
	21-25	2
	26-30	3
	>30	4
Nasal discharge	None	0
	Serous	1
	Mucopurulent	3
Abdominal lift	None	0
	Mild (perceptible, heave line)	1
	Pronounced (abdomen, thorax and anal movement)	3
Nasal flaring	None	0
	Present	1
Tracheal sounds	Normal (tubular sound)	0
	Increase in intensity	1
	Mucus movement	3
Bronchial tones	Normal	0
	Audible ventral and dorsal sounds	2
Crackles	None	0
	Present	2
Wheezes	None	0
	Present	2
Cough	None	0
	Inducible by tracheal massage	1
	Intermittent	2
	Paroxysmal	3
Total (maximum)		23

Lung function testing

Pulmonary mechanics were performed as previously described [7] at the beginning of the placebo week (day 0) and/or at days 0, 7, 14, and 21 of each cycle of treatment (see Fig 1). Airflow rates and ΔP_L were obtained as previously described [7] using a heated pneumotachograph connected to a face-tight mask and an esophageal catheter with an inflatable balloon on its end, respectively. Pulmonary resistance (R_L) and elastance (E_L) were obtained by means of a multiple regression equation for the single compartment model of the lung: $\Delta P_L = (E_L \cdot V) + (R_L \cdot v) + K$, where V is the lung volume, v is the airflow, and K is the transpulmonary end-expiratory pressure (constant).

Bronchoreversibility test

Pulmonary mechanics were performed before and 20 to 30 min after the administration of a muscarinic antagonist. In study I, atropine (0.02 mg/kg i.v.) was administered once at day 21 of the fourth cycle of treatment. In study II, both atropine and N-butyl-scopolamine were administered using a cross over design at days 28 and 35 of the fourth cycle of treatment (the results of this trial are now published [8]). In study III, N-butyl-scopolamine (0.3 mg/kg i.v.) was administered once at day 21 of the fourth cycle of treatment.

Blood samples

Blood was collected for CBC, biochemistry and cortisol levels assessment as described in Figure 1.

Samples for haematology/biochemistry and cortisol were collected in the morning between 06:00-08:00 prior to the first treatment dose of the day. Samples for CBC and biochemistry were collected in EDTA and in dry tubes by sterile venipuncture during the conditioning, at day 0 of the placebo weeks and/or at days 0, 14 and 21 of each cycle of treatment (that is, before and after each treatment period, and one week after the end of the treatment). Serum cortisol was measured on days 0, 3, 5, 7, 10, 14, 17 and 21 of each cycle of treatment (6 measurements during the treatment period and 2 during the post-treatment week). Blood collected for cortisol level measurements was allowed to coagulate for 45 min before being spun, aliquoted and placed on dry ice within 120 min from collection. It was then stored at -80°C. All samples were analysed together at the end of each study by solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite®, Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA). The minimum reading in the test performed is 10.2 nmol/L; values smaller than 10.2 are shown as 10.2 nmol/L.

Statistical analysis

Statistical analysis was performed with SAS v.9.3 (Cary, NC, USA). In studies I and II, data obtained during the placebo phase were analysed separately using paired t-test. Two mixed linear models with horses as random factor were used to investigate the effect of treatment order (treatment and treatment order as fixed factors, interaction between the two parameters studied) and of the cycle of treatment (that is, whether initial values differed among treatments; fixed factor, no interaction studied) the parameters studied. A linear mixed model was used to assess the effect of time, treatment, and their interaction in each study, with horse ID as a random factor (treatment and time as within-subject factors, 4 levels each) and fixed effects of treatment, time and the interaction between treatment and time. We performed visual checks of model assumptions using residual values. *A priori* contrasts were performed to compare means of the same treatment at different time points and to compare means of different treatments at the same time point. The alpha level for each contrast was adjusted downward with the sequential Benjamini-Hochberg procedure. Cochran-Mantel-Haenszel test was employed to analyse ordinal data (breathing effort score and weighted clinical score) using the horse ID as stratum and assuming that treatment order had negligible effect. The association between clinical scores and lung function parameters were evaluated using linear regression test. Paired t-tests were employed to evaluate bronchoreversibility data.

Results

Horses

Sixteen horses were studied. Among them, 2 horses participated in study I and II, 2 horses participated in study II and III, and another 2 horses participated in all 3 studies. Baseline details of the horses enrolled in each study are provided in the table below. The severity of airway obstruction ranged from mild to severe in individual horses before the placebo and each treatment phase in all studies. One horse was enrolled in study III despite a history of previous laminitis because of the unavailability of other horses. During study III, a horse developed life-threatening airway obstruction on day 7 of treatment phase with ciclesonide 2700 µg twice daily. As dictated by rescue criteria of the study protocol, mouldy hay challenge was suspended for 6 weeks for this animal and it was excluded from all analysis (n = 7 in study III).

Reproducibility of experimentally-induced personalised controlled equine asthma exacerbations

No effect of treatment order was noticed for any of the parameters studied. Values of lung function and clinical scores at day 0 were not affected by the treatment cycle in any of the studies reported, meaning that, within the same study, data at days 0 of different cycles of treatment were not different. These data suggest

that the effects of experimentally-induced personalised controlled equine asthma exacerbations on lung function and clinical score are reproducible across subjects within a short period of time.

Significant variations among cycles of treatment were observed only during study II. Serum cortisol values were significantly lower in all horses during the first cycle of treatment compared to the second and third ones ($p = 0.01$ and $p = 0.048$, respectively). Overall, PCV and total proteins significantly decreased in all horses during the fourth cycle of treatment compared to the first ($p = 0.006$ and $p = 0.02$, respectively) and second ($p = 0.02$ and $p = 0.03$) ones. Similarly, WBC significantly decreased during the fourth cycle of treatment compared to the first one ($p = 0.02$). All values remained within normal ranges, however.

Adverse events

In study I, AE were most frequently represented by ringworm lesions, observed in 7/8 horses with onset during the washout week of the first cycle of treatment and the first week of the second cycle of treatment. Two episodes of alopecia were also reported, chronologically not associated with ringworms, of which one during ciclesonide treatment at 900 µg and the other during conditioning. Hyperthermia was observed in concomitance with severe bronchospasm in 2 horses (breathing effort score 8) during the washout week of the first cycle of treatment. Four episodes of fever were reported, of which one during ciclesonide treatment at the lower dose of 450 µg, and two occurring concomitantly with foot abscess. Two horses suffered of corneal ulcers (one horse had bilateral ulcers), but none of them developed during ciclesonide treatment. The horse with bilateral ulcers showed prolonged ocular scratching after ulcers healing. Two horses had foot abscesses: one during conditioning and the other had repetitive ($n = 3$) abscesses of the right front foot during the post-treatment week of dexamethasone, the post-treatment week of ciclesonide 1800 µg, and during the treatment with ciclesonide 450 µg). Thrush (hoof bacterial infection) was observed in one horse not suffering from hoof abscesses during the post-treatment week of ciclesonide 900 µg. An episode of colic also occurred during dexamethasone treatment.

In study II, lesions due to eye scratching were observed in one horse treated with dexamethasone, while generalised scratching was observed in a horse during ciclesonide treatment at 2700 µg (nozzle A).

In study III, AE consisted mainly of pruritus and epiphora, respectively reported in 2 and 5 horses during ciclesonide treatments (2 and 6 observations) and in 1 and 2 horses (2 observations for both) during placebo/post-treatment/washout phases. Hyperthermia was observed in one horse during placebo cycle of treatment, while fever was found in 2 horses on ciclesonide treatment 2700 µg BID and 3712.5 µg in the morning, once in association with laboured breathing. Laboured breathing in absence of increased body

temperature was observed once during placebo cycle of treatment. Isolated episodes of dermatitis (one horse, onset during ciclesonide treatment 3712.5 µg morning and lasting for 5 weeks), colic (one horse, on placebo), and hoof bacterial infection (one horse, washout week of ciclesonide treatment 3712.5 µg evening) were also noticed.

Discussion

Adverse events

Adverse events were recorded during the 3 studies and defined as any changes in the horse health status requiring the attention of the attending veterinarian. Due to our definition of AE, however, it is likely that also AE not connected to ciclesonide administration or the way of administering it have been recorded. To this regard, ringworm lesions were observed in 7/8 horses during study I with onset during a two-week period in all animals, but in none of the animals included in studies II and III, although higher doses were tested. Given the ability of ringworm spores to persist in the environment for years, the highly contagious nature of ringworm infection, and its incubation time (9-15 days) [11], it is likely that an outbreak occurred in concomitance of study I, independently of the treatment administered.

Epiphora/ocular discharge and corneal ulcers were frequently observed in all three studies. In light of the role of airborne organic dust in the induction of equine asthma exacerbations, it is likely that the horses were exposed to high levels of suspended organic dust during our experimental procedures, causing ocular irritation or even corneal lesions. During study III, when the highest doses were tested, the number of horses developing epiphora during ciclesonide treatment was more than two-fold that observed in absence of ciclesonide, questioning whether an association with this treatment might somehow be present. Given the low number of horses studied, the study design with the same horses repeatedly exposed to the mouldy environment, and the short duration of washout phase, statistical analysis on the association of specific AE with ciclesonide treatment was not attempted. The ocular safety of ICS is a concern that might have been not adequately addressed to date, even in human patients [12,13]. Ciclesonide metabolism and safety profile however should prevent/reduce these effects. Further studies will have to address this specific point before a causal relationship can be established.

Foot problems (abscesses and thrush) occurred during study I and III. All except one horse had a previous history of foot disorders. The remaining horse had a foot abscess during conditioning, thus before receiving any dose of corticosteroids. Of note, horses were kept unshod during the study periods, and paddock soil

might have been moist after rainy days. These factors might have contributed to abscess development in the horses studied.

Finally, one horse developed a life-threatening airway obstruction on day 7 of ciclesonide treatment 2700 µg treatment and was then excluded from study III. Other animals had severe airway obstruction occasionally observed during our studies. Ciclesonide-induced bronchospasm is a potential side effect of the drug recognised in human asthma patients [9]. However, this is unlikely to have occurred in our horses, as the effect was not associated with administration of ciclesonide but likely resulted from temporarily increased antigen burden or concomitant change in environmental conditions [10].

Baseline details of the horses enrolled in each study.

	Study I	Study II	Study III
Horses, n	8	8	7
Sex, mares/gelding	5/3	5/3	5/2
Age, years	19 ± 4	16 ± 6	15 ± 5
Weight, kg	459 ± 106	458 ± 105	480 ± 42
ΔP_L, cmH_2O	40.9 ± 17.2	47.1 ± 18.6	29.0 ± 8.6
R_L, $cmH_2O/L/s$	2.8 ± 1.0	2.7 ± 0.6	2.3 ± 0.5
E_L, cmH_20/L	3.9 ± 2.4	5.1 ± 2.3	2.7 ± 1.4
Breathing effort score	7.1 ± 0.4	-	-
Weighted clinical score (WCS)	-	15.1 ± 2.2	14.3 ± 1.9
Serum cortisol, nmol/L	67 ± 35	80 ± 20	93 ± 12
Hematocrit, %	36 ± 4	37 ± 5	35 ± 3
Total protein, g/L	73 ± 7	74 ± 5	71 ± 4
Leukocytes, $\cdot 10^9/L$	8.1 ± 0.8	7.8 ± 1.7	7.3 ± 1.0
Segmented neutrophils, $\cdot 10^9/L$	5.5 ± 1.3	5.3 ± 1.3	4.5 ± 1.0
Lymphocytes, $\cdot 10^9/L$	2.1 ± 0.6	2.0 ± 0.6	2.4 ± 0.4

Data are expressed as mean ± s.d. and refer to day 0 of the treatment cycle 1.

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