

Corticosteroids and Antigen Avoidance Decrease Airway Smooth Muscle Mass in an Equine Asthma Model

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Recent studies suggest that airway smooth muscle remodeling is an early event in the course of asthma. Little is known of the effects of long-term antigen avoidance and inhaled corticosteroids on chronically established airway remodeling. We sought to measure the effects of inhaled corticosteroids and antigen avoidance on airway remodeling in the peripheral airways of horses with heaves, a naturally occurring asthma-like disease. Heaves-affected adult horses with ongoing airway inflammation and bronchoconstriction were treated with fluticasone propionate (with and without concurrent antigen avoidance) ($n = 6$) or with antigen avoidance alone ($n = 5$). Lung function and bronchoalveolar lavage were performed at multiple time points, and peripheral lung biopsies were collected before and after 6 and 12 months of treatment. Lung function improved more quickly with inhaled corticosteroids, but eventually normalized in both groups. Inflammation was better controlled with antigen avoidance. During the study period, corrected smooth muscle mass decreased from $12.1 \pm 2.8 \times 10^{-3}$ and $11.3 \pm 1.2 \times 10^{-3}$ to $8.3 \pm 1.4 \times 10^{-3}$ and $7.9 \pm 1.0 \times 10^{-3}$ in the antigen avoidance and fluticasone groups, respectively ($P = 0.03$). At 6 months, smooth muscle mass was significantly smaller compared with baseline only in the fluticasone-treated animals. The subepithelial collagen area was lower at 12 months than at baseline in both groups. During the study period, airway smooth muscle remodeling decreased by approximately 30% in both groups, although the decrease was faster in horses receiving inhaled corticosteroids. Inhaled corticosteroids may accelerate the reversal of smooth muscle remodeling, even if airway inflammation is better controlled with antigen avoidance.

Keywords: airway remodeling; smooth muscle; inhaled glucocorticosteroids; fluticasone; animal model

Airway remodeling in asthma is characterized by a thickening of the airway wall, in part because of an increase in airway smooth muscle (ASM) and subepithelial collagen deposition (reviewed by Sumi and Hamid) (1). Airway smooth muscle thickening

contributes to the loss of lung function and the development of persistent airflow limitation in asthma by reducing baseline airway caliber and exaggerating airway narrowing during bronchospasm (2–4). The finding that ASM thickness is related to the severity and not the duration of asthma (3), combined with the increased ASM found in children with asthma (5), indicates that ASM remodeling may constitute an early event in the course of the disease, and may be stable over extended periods of time. However, evidence also exists that ASM remodeling is dynamic, even in long-established severe asthma (6). Therefore, investigating the effects of intervention late in the remodeling process is important for adult patients with asthma, because smooth muscle behavior could differ from what is observed in acutely induced ASM remodeling models. Unfortunately, the reversibility of remodeling in human subjects is difficult to study for ethical reasons and the limitations imposed by sampling techniques. Recently, positive effects of a short course of inhaled corticosteroids (ICS) on smooth muscle were described in patients with asthma (7). Moreover, indirect evidence exists of possible ASM remodeling reversibility with antigen avoidance in work-related asthma (8). Subepithelial fibrosis in asthma is believed to be secondary to the activation of fibroblasts and other mesenchymal cells, leading to an increase in the deposition of extracellular matrix proteins, mostly of collagen fibers (1, 9). Subepithelial collagen is considered poorly responsive to intervention, although some reversibility has been reported (10, 11).

Heaves is a naturally occurring disease of adult horses that shares numerous similarities with asthma, including reversible bronchoconstriction and airway inflammation when susceptible horses inhale antigens of their environment (12, 13). Coughing, wheezing, and exercise intolerance are present during clinical exacerbations, and can be controlled by antigen avoidance (14, 15) or corticosteroids and bronchodilators (12, 16). Despite links to Th2 cytokines in some studies, intraluminal inflammation is predominantly neutrophilic, as may also be observed in some types of asthma (17, 18). Airway remodeling in heaves includes epithelial detachment and regeneration, goblet-cell hyperplasia, and increased bronchial and bronchiolar smooth muscle (15, 19, 20). Collagen also contributes to increased airway wall thickness (21), although the specific subtypes that are altered remain unclear (22, 23). We recently observed that chronic ASM remodeling is maintained in a dynamic equilibrium with an increased myocyte turnover (15), not unlike the findings in severe asthma (6). This suggests that an intervention affecting the proliferation of ASM could reverse ASM remodeling, even if chronically established.

This study was designed to evaluate the effects of long-term treatment with inhaled corticosteroids and antigen avoidance in an equine model of asthma. We hypothesized that (1) chronic ASM remodeling is partly reversible with long-term ICS therapy or prolonged antigen avoidance, and (2) a combination of both approaches would lead to a greater decrease in ASM. We also hypothesized that the subepithelial collagen area and

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epithelial detachment would decrease with intervention. To test these hypotheses, we treated symptomatic heaves-affected horses with ICS (with continuous antigen exposure for 6 months, and then with the addition of antigen avoidance) or with antigen avoidance alone. Lung function, inflammation, and ASM remodeling in peripheral lung biopsies were measured before, during, and at the end of treatment.

MATERIALS AND METHODS

Additional information is available in the online supplement.

Experimental Design and Animals

Eleven adult heaves-affected horses were housed indoors and exposed to hay until they all showed clinical signs associated with airflow limitation (baseline). Treatment consisted of strict antigen avoidance as sole therapy for five horses. The remaining six horses were treated with ICS. They remained exposed to hay for the first 6 months (ICS with antigen exposure), and then were turned out to pasture for the second half of the study (ICS combined with antigen avoidance). Pulmonary function, bronchoalveolar lavage, and peripheral lung biopsies were collected when horses had been exposed to hay for more than 1 month (baseline) and after 6 and 12 months of treatment. Additional pulmonary function and bronchoalveolar lavage data were collected at 1 and 7 months.

Treatments

Antigen avoidance consisted of keeping horses in the pasture and supplementing them with a pelleted diet. In the ICS group, fluticasone propionate (Flovent HFA; GlaxoSmithKline, Montreal, PQ, Canada) was administered at a dose of 2,000 μg every 12 hours for a month, and then doses were adjusted to control clinical signs (2,000–3,000 μg every 12–24 hours). Two horses received 2,000 μg every 24 hours from Month 1, whereas the others required higher or more frequent administration during the first 6 months.

Lung Biopsies via Thoracoscopy

Peripheral lung tissue was harvested via thoracoscopy in the caudodorsal region of the lung on standing, sedated animals (further details are available in the online supplement).

Immunostaining, Enzymatic Labeling, and Picrosirius-Red Staining

Details are provided in the online supplement.

Morphometry

The ASM area of airways in cross section was measured using Image-Pro Plus software (MediaCybernetics, Carlsbad, CA). The ASM area (median number of airways per animal, 10; range, 5–17), proliferating cell nuclear antigen positive (PCNA⁺) myocytes (median, 5; range, 3–5) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling positive (TUNEL⁺) myocytes (median, 5; range, 2–5) were corrected by the internal perimeter squared to account for variation in airway size (24). Measurements were performed by one investigator, blinded to study groups and time points. Additional information, including subepithelial collagen area (i.e., collagen between the epithelium and the ASM layer) and measurements of epithelial detachment, is available in the online supplement.

Pulmonary Function

Details are provided in the online supplement.

Bronchoalveolar Lavage Fluid Collection

Details are provided in the online supplement.

mRNA Expression of Bronchoalveolar Lavage Cells

Details are provided in the online supplement.

Histological Scoring

Details are provided in the online supplement.

Statistical Analysis

Physiologic, bronchoalveolar (BAL), and morphometric data were analyzed by a repeated-measures linear model with “group” as a between-subjects factor and “time” as within-subject factor. Within-group and between-groups differences were further evaluated using *a priori* contrasts. Prebronchodilation and postbronchodilation data were analyzed separately with the same model. Inflammation scores were analyzed with the Cochran–Mantel–Haenszel test for ordinal data. Agreement between biopsy scoring was evaluated by weighted κ . $P \leq 0.05$ was considered statistically significant. See the online supplement for further details.

RESULTS

Animals

Age, weight, and proportion of mares were not statistically different between groups (mean age $\pm$ SD of the antigen avoidance group, 17.4 ± 3.6 ; mean age $\pm$ SD of the ICS group, 18.7 ± 2.2 years; weights, 483 ± 40 kg for the antigen avoidance group, and 485 ± 60 kg for the ICS group; 3/5 mares in the antigen avoidance group, and 4/6 mares in the ICS group). In the ICS group, one horse was not available for lung function measurements and BAL at 7 months, and for another horse, postbronchodilator measurements were not obtained for technical reasons.

Airway Smooth Muscle

Airway smooth muscle mass, normalized for airway size, was similar in both groups at baseline (antigen avoidance, mean $\pm$ SD, $12.13 \pm 6.20 \times 10^{-3}$; ICS, mean $\pm$ SD, $11.26 \pm 2.91 \times 10^{-3}$) and significantly decreased over the 12-month period with both treatment regimens (antigen avoidance, mean $\pm$ SD, $8.33 \pm 3.12 \times 10^{-3}$; ICS, mean $\pm$ SD, $7.86 \pm 2.42 \times 10^{-3}$) ($P = 0.03$ in both groups) (Figure 1A). A positive correlation was observed between ASM mass at baseline and at 12 months ($r = 0.74$, $P = 0.006$). In the antigen avoidance group, ASM decreased gradually during the period of the study, reaching a change of $27.7\% \pm 9.6\%$ at 12 months. Interestingly, a similar maximal improvement was also seen in the ICS group, but it was reached by 6 months, with no further decrease during the second half of the study ($27.7\% \pm 13.3\%$ at 6 months, $P = 0.048$; $28.4\% \pm 9.7\%$ at 12 months). An analysis of individual data shows that ASM had decreased in eight horses at 6 months, and in all but one at 12 months. No significant change over time was evident in the density of ASM nuclei (Figure 1B), and a significant decrease in ASM nuclei corrected by the internal perimeter squared was observed in the ICS group (Figure 1C). A positive correlation between ASM mass and elastance was observed at 12 months ($r = 0.63$, $P = 0.037$).

Proliferation and Apoptosis of Airway Smooth Muscle

No significant change was observed in PCNA⁺ or TUNEL⁺ myocytes between the beginning and end of the study in either group (Figures 1D and E). However, in the ICS group, a decrease in PCNA almost reached significance at 6 months ($P = 0.07$), corresponding to the steepest decline in ASM mass observed in the study.

Pulmonary Function

Pulmonary function data are shown in Figure 2A. After antigen exposure (baseline), both groups demonstrated a similar degree

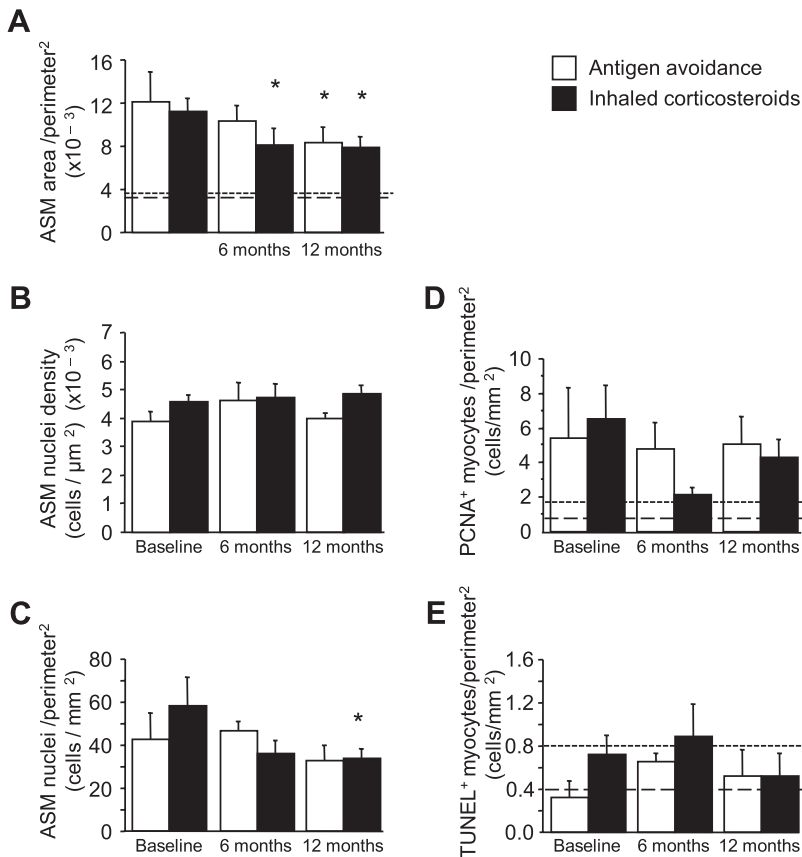


Figure 1. Airway smooth muscle remodeling. Airway smooth muscle (ASM) area (A), density of ASM nuclei (ASM nuclei/ASM area) (B), ASM nuclei (C), proliferating cell nuclear antigen positive (PCNA⁺) myocytes (D), and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling positive (TUNEL⁺) myocytes (E) (corrected for the internal perimeter squared) of heaves-affected horses after antigen exposure (Baseline) and after 6 and 12 months of treatment with antigen avoidance alone (open bars) ($n = 5$) or inhaled corticosteroids (solid bars) ($n = 6$). Antigen exposure persisted for the first 6 months of treatment in the ICS group. Values represent means $\pm$ SEM. Dashed lines represent historical mean values in control horses. Lower dashed lines represent data from Herszberg and colleagues in 2006 (19); upper dashed lines represent data from Leclerc and colleagues in 2011 (15). *Different from baseline, $P \leq 0.05$.

of airflow limitation, with individual resistance values ranging from 2.7–3.8 cm H₂O/L/second and 2.1–4.4 cm H₂O/L/second, and elastance from 2.1–7.0 cm H₂O/L and 2.2–6.0 cm H₂O/L, in the antigen avoidance and ICS groups, respectively (reference range: resistance, < 1 cm H₂O/L/second; elastance, < 1 cm H₂O/L) (25). Pulmonary elastance rapidly decreased and returned to values close to normal in both groups (significantly different from baseline at all time points). The decrease in resistance was more pronounced during the first month in the ICS group. This improvement was faster but not complete, because an additional decrease was observed when ICS-treated, antigen-exposed horses joined the antigen avoidance group at 6 months. Resistance was significantly lower after bronchodilator administration after 12 months of treatment (Figure 2B), suggesting residual bronchoconstriction in both groups. The only horse that did not show a decrease in resistance also demonstrated the lowest prebronchodilator value.

Subepithelial Collagen Content and Epithelial Detachment

Subepithelial collagen area was measured in nine horses (five in the antigen avoidance group, and four in the ICS group), based on tissue availability. Corrected collagen area decreased from $19.32 \pm 17.68 \times 10^{-3}$ and $24.67 \pm 4.48 \times 10^{-3}$ (mean $\pm$ SD) for the antigen avoidance and ICS groups, respectively, to $9.89 \pm 8.13 \times 10^{-3}$ and $11.47 \pm 9.20 \times 10^{-3}$ after 12 months (Figure 3A). A large variation in baseline collagen area values was observed in the antigen avoidance group, and no significant change over time was evident when all time points were considered. When values at baseline and at 12 months were analyzed separately, a marginally significant decrease was observed in both groups ($P = 0.05$ and 0.01 for antigen avoidance and ICS, respectively, according to one-tailed t test). Moreover, an 11.7–78.3%

decrease in collagen area was evident for eight of the nine horses, and one horse in the antigen avoidance group exhibited a 3.6% increase. Subepithelial collagen areas at baseline and at 12 months were positively correlated ($r = 0.77$, $P = 0.012$), as was collagen and elastance at baseline ($r = 0.74$, $P = 0.021$). Epithelial detachment was significantly lower at 6 months compared with baseline in the antigen avoidance group (Figure 3B).

Bronchoalveolar Lavage Fluid

As a result of the progressive resolution of bronchospasm, the percentage of return volume increased during the experiment, affecting absolute cell counts. Analysis of the differential cell count shows that, as expected in horses with heaves, antigen exposure led to a high percentage of neutrophils in baseline BAL. In the antigen avoidance group, neutrophils had returned to normal (< 10%) by Month 6. In the fluticasone group, however, pulmonary neutrophilia only decreased after antigen exposure ceased, with a significant drop observed between Months 6 and 7 (Figure 4).

mRNA Expression of BAL Cells

In both groups, a significant decrease in IL-8 mRNA occurred at 6, 7, and 12 months compared with baseline (Figure 5A). The decrease was more pronounced in the antigen avoidance group, where IL-8 was significantly lower at 6 and 7 months, compared with the ICS group. A transient increase in TNF- α was observed in the antigen avoidance group at 1 month, and a group-time interaction was observed with higher concentrations of TNF- α in the ICS group at 6, 7, and 12 months, without a significant change from baseline. No change in transforming growth factor (TGF)- β expression was observed over time (Figure 5C).

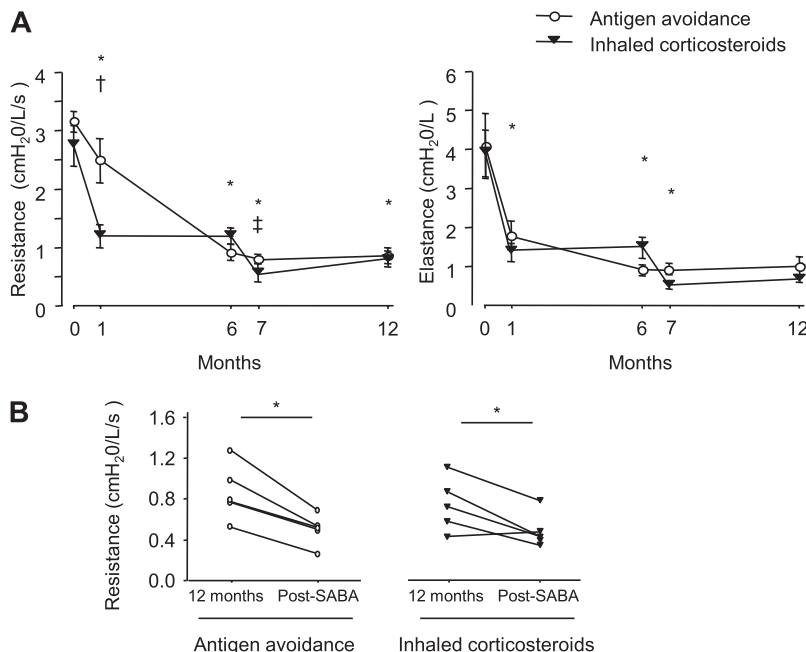


Figure 2. Pulmonary function. (A) Pulmonary resistance and elastance of heaves-affected horses after antigen exposure (Baseline) and after 1, 6, 7, and 12 months of treatment with antigen avoidance alone (open circles; $n = 5$) or inhaled corticosteroids (ICS, solid triangles; $n = 6$). Antigen exposure persisted for the first 6 months of treatment in the ICS group; antigen avoidance was combined with ICS from Months 6–12. Values represent means $\pm$ SEM. *Different from baseline (both groups). †Different between groups at the same time point. ‡Different from 6 months (ICS only), $P \leq 0.05$. (B) Pulmonary resistance of heaves-affected horses after 12 months of treatment with antigen avoidance alone (open circles; $n = 5$) or inhaled corticosteroids (solid triangles; $n = 5$), and 30 minutes after the administration of a short-acting bronchodilator administration (Post-SABA). *Different between pre-SABA and post-SABA administration, $P \leq 0.01$.

Inflammation Scores

Tissue quality was deemed excellent, very good, good, and inadequate in 20, 10, 3, and 0 biopsies, respectively. No significant changes were observed in the bronchial/endobronchial and interstitial inflammation scores. For the peribronchial inflammation and intraluminal mucus scores, an overall time effect ($P = 0.03$) was evident only in the antigen avoidance group, with marginally lower scores at 12 months (Figure 6).

DISCUSSION

In the present study, we have examined the effects of inhaled corticosteroids and strict antigen avoidance on the possible reversibility of ASM remodeling. We also investigated their effects on subepithelial collagen area and epithelial detachment. The results indicate that chronic ASM remodeling is partly reversible, even in animals that have experienced multiple episodes of antigen exposure and airway obstruction over a period of many years. A reduction in ASM remodeling was hastened by ICS, but the maximal improvement was of the same magnitude in both groups at the end of the 12-month study period. A cumulative effect of ICS and antigen avoidance in further reducing ASM remodeling, as we had hypothesized, was not observed. These results suggest that a component of ASM remodeling may be refractory to intervention. In addition, the data support a potential reversibility of subepithelial collagen remodeling.

Inhaled Corticosteroids Accelerate ASM Decrease Compared with Antigen Avoidance Alone

Inhaled corticosteroids accelerated the decrease in ASM remodeling, and almost all of the ASM reduction occurred during the first 6 months of the study in this group. Inhaled corticosteroids, however, did not affect overall improvement during a 1-year period compared with antigen avoidance alone, and the improvement remained modest ($\sim 30\%$) in both groups, with mean values remaining approximately twice those of historical healthy control animals from two previous studies (15, 19). These results contrast with the complete reversibility of induced airway remodeling observed in rodent models when corticosteroids were administered early (26), or were combined with

other anti-inflammatory medication (27). The modest decrease in ASM we observed may nonetheless be clinically relevant, because mathematical modeling indicates that variation in ASM mass leads to much greater changes (many fold) in airway obstruction during bronchoconstriction and in airway hyperresponsiveness (28).

Studies addressing the reversibility of ASM remodeling with corticosteroids in patients with asthma are scarce. In one study, a substantial (60%) decrease in the percentage of transbronchial biopsies occupied by smooth muscle was observed after only a 6-week treatment with ICS (7). Although corrections for airway size and comparisons with control samples were not possible, this study nonetheless suggests that ASM mass can be affected by ICS. Similarly, we observed a significant reduction of ASM in the first 6 months of ICS treatment, and because intermediary time points were not available, we cannot exclude the possibility that most of the decrease took place soon

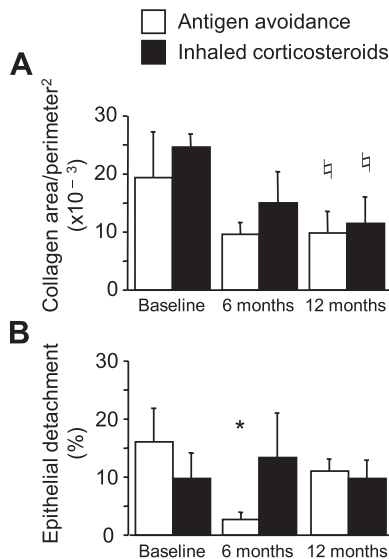


Figure 3. Subepithelial collagen area and epithelial detachment. Subepithelial collagen area (collagen present between the epithelium and the ASM layer) corrected for the internal perimeter squared (A) and epithelial detachment (B) of heaves-affected horses after antigen exposure (Baseline) and after 6 and 12 months of treatment with antigen avoidance alone (open bars; $n = 5$) or inhaled corticosteroids (solid bars; A, $n = 4$; B, $n = 6$). Antigen exposure persisted for the first 6 months of treatment in the ICS group. Values represent means $\pm$ SEM. Trapezoid: Different from baseline according to one-tailed t test. Asterisk: Different from baseline. $P \leq 0.05$.

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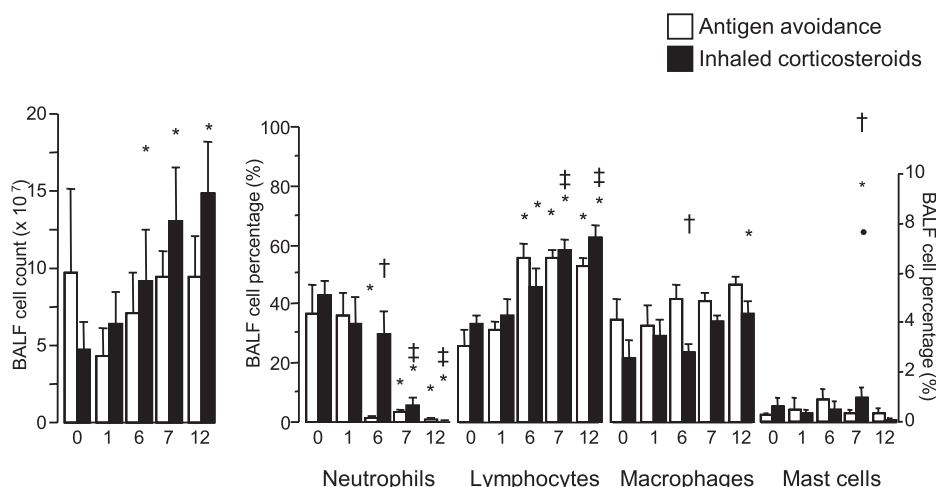


Figure 4. Bronchoalveolar lavage (BAL) cytology. Bronchoalveolar lavage fluid (BALF) total cell count and differential cell count of heaves-affected horses after antigen exposure (baseline = Month 0) and after 1, 6, 7, and 12 months of treatment with antigen avoidance alone (open bars; $n = 5$) or inhaled corticosteroids (solid bars; $n = 6$). Eosinophils were absent from all but six BAL samples, and are not displayed. Antigen exposure persisted for the first 6 months of treatment in the ICS group. Values represent means $\pm$ SEM. *Different from baseline in the same group. †Different from antigen avoidance at the same time point. ‡Different from 6 months in the same group. Solid dot represents outlier. $P \leq 0.05$.

after the initiation of therapy. This accelerated decrease is intriguing and unexpected, because it occurred despite continued antigen exposure and evidence of persistent airway inflammation. Airway remodeling is believed to be a consequence of chronic inflammation (29), therefore making it only logical to hypothesize that a reversal of remodeling would be achieved by controlling inflammation. To the contrary, inflammation was better controlled by antigen avoidance than with ICS, when looking at BAL differential cell count, cytokine expression by BAL cells, histological changes in peripheral airways, or intraluminal mucus accumulation (Figures 4, 5, 6C, and 6D). Other markers of inflammation not measured in our study may have been suppressed by ICS, or else ICS may have affected ASM remodeling mainly through a direct anti-inflammatory effect on myocytes (30) or epithelial cells (31). Of note, a significant decrease in IL-8 occurred during the first half of the study in both groups of horses. Even if it was not as marked with ICS as with antigen avoidance, this decrease nevertheless supports the anti-inflammatory effect of ICS under conditions of continuous exposure, and this effect may be stronger at the level of structural cells. ICS treatment was also not associated with a decrease in TGF- β , a growth factor not only linked to collagen remodeling but also to ASM remodeling (32). This is consistent with findings in some patients with asthma treated with corticosteroids (7, 33), but in contrast to the decrease observed with a combination of ICS and long-acting β_2 -agonists (34). In our study, no change of TGF- β occurred over time in either group, suggesting that a change in BAL cell expression of this growth factor is not the main mechanism by which remodeling decreases with ICS or antigen avoidance. TGF- β may not play an important role in horses, or as seen in asthma, its pattern of expression and main cellular source may be complex and variable.

Noninflammatory mechanisms of remodeling, such as mechanical stimuli, have also been described (35). Corticosteroids could affect ASM remodeling independently of their anti-inflammatory effect, by reducing the mechanical stress associated with bronchospasm or by decreasing myocyte proliferation (36). In this study, the steepest decrease in ASM, observed in the first 6 months of treatment with ICS, was associated with an almost significant decrease of myocyte proliferation marker at 6 months ($P = 0.07$) (Figure 1D). An increase in apoptosis was not observed in either group or at any time point, making it an unlikely mechanism of decreased remodeling. Myocyte size could not be measured directly, but the lack of variation in the number of nuclei per ASM area

(Figure 1B) suggests that the decrease in mass is not primarily attributable to a decrease in cell size or in extracellular matrix between the myocytes. This suggests a decrease in cell number, and argues that the increase in area of ASM is attributable at least in part to hyperplasia, which is consistent with previous work in heaves-affected horses (15) and with some data on human asthma (37–39). We therefore looked at the number of nuclei, corrected by the internal perimeter, and partly confirmed this hypothesis (Figure 1C). Although not definitive, these findings support further studies using stereology to quantify changes in myocyte size and their surrounding extracellular matrix, as recently described by James and colleagues (38), over time and with different treatments. Finally, the lack of further improvement in ASM mass during the second half of the study could be explained by a partly irreversible increase in mass, therefore making a synergistic effect of ICS and antigen avoidance unlikely to be observed with this experimental design.

Subepithelial Collagen Content and Epithelial Detachment

Collagen remodeling is often considered poorly responsive to intervention (7, 33), although a modest positive effect on subepithelial fibrosis has been reported with long-term treatment (10), or with prolonged antigen avoidance in occupational asthma (11). Moreover, *in vitro* evidence exists that fibroblasts are not intrinsically resistant to corticosteroids (35), and collagen turnover is higher in the lung (more than 10% per day) than in the skin of adult rats (40). Despite missing data in the ICS group, these findings strongly suggest that subepithelial collagen area can decrease over time with strict antigen avoidance or with ICS. Subepithelial collagen may exert a partly protective effect to counterbalance ASM remodeling by attenuating its shortening (35). A correlation between ASM and collagen area or in their respective percentage of reduction would have been expected, but it was not the case here. The reduced collagen content was not associated with decreased TGF- β expression by BAL cells. The quantification of TGF- β expression at the tissue level could be of the interest in elucidating its role in collagen and ASM remodeling. In asthma, epithelial detachment is thought to involve more than artifacts, representing instead a loss of epithelial integrity and a weakening of adherence molecules that are affected by inflammation (35). The decrease in epithelial detachment with antigen avoidance is interesting and may be attributable to the decrease in inflammation observed, but it was only significant at 6 months, and we are unsure whether it reflects a real reduction of epithelial fragility.

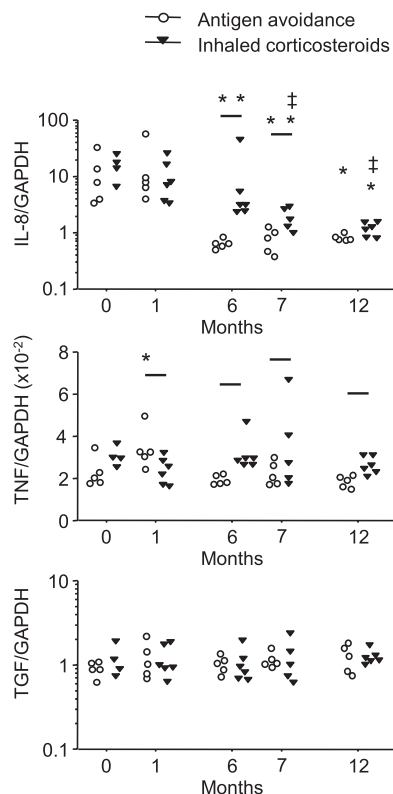


Figure 5. Bronchoalveolar lavage cell gene expression of IL-8, TNF- α , and transforming growth factor- β (TGF- β), relative to the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in heaves-affected horses after antigen exposure (baseline = Month 0) and after 1, 6, 7, and 12 months of treatment with antigen avoidance alone (open circles; $n = 5$) or inhaled corticosteroids (solid triangles; $n = 6$). Antigen exposure persisted for the first 6 months of treatment in the ICS group. *Different from baseline in the same group. †Different from baseline in the same group. ‡Different from baseline in the same group. Dash represents difference between groups at one time point. ‡Different from 6 months in the same group. $P \leq 0.05$.

Correlation between Remodeling and Lung Function Parameters

The correlation between peripheral ASM and lung function after 12 months of ICS or strict antigen avoidance suggests a role of ASM remodeling in the residual small airway obstruction observed. Small airway ASM remodeling is associated with increased elastance and peripheral tissue damping in some models of asthma (41), and with elevated elastance and a heterogeneous pattern of constriction in a human lung model (42). The correlation between high subepithelial collagen area and elastance at baseline (when horses are symptomatic) supports the role of subepithelial collagen in reducing airway distensibility and lung compliance (43), and this phenomenon could be accentuated during bronchospasm.

Effects of Antigen Avoidance on Inflammation, Function, and ASM Remodeling

Current recommendations for asthma therapy include environmental control and allergen avoidance strategies (44). Complete avoidance is difficult to achieve, but when implemented, improvements in lung function (45) and inflammation (46) are observed. Little is currently known about the effects of antigen avoidance as sole therapy in human subjects with asthma (47, 48) because of ethical considerations. However, in occupational asthma where antigen avoidance is more easily achieved, work cessation is beneficial to lung function (49), inflammation (50), and possibly remodeling. Sumi and colleagues found normal ASM content in bronchial biopsies obtained decades after patients with asthma had been removed from their work environments (8). That study, however, did not show that ASM remodeling was present in these workers initially, because biopsies were not performed at the time of diagnosis. In the present study, antigen avoidance alone was associated with a more rapid control of BAL inflammation than was ICS, and antigen avoidance was also associated with modest effects on biopsy inflammation scores at 12 months. Further underlying the importance of antigen avoidance, airway inflammation normalized only when antigen avoidance was added to ICS in this group, and it further decreased IL-8 expression and improved airway function in those horses (with a significant decrease in resistance and IL-8 between 6 and 7 months; Figures 2 and 5). In summary, despite showing a slower effect on ASM remodeling, this study highlights the importance of antigen avoidance in the control of antigen-induced airway inflammation and obstruction.

Different Effects of ICS and Antigen Avoidance on Lung Function

Although not the primary goal of this study, a few aspects of the effects of ICS and antigen avoidance on pulmonary function are of interest. First, ICS-treated animals demonstrated a faster improvement of lung resistance than horses in the antigen avoidance group, despite their continual exposure to the environmental antigens that triggered airflow limitation in the first place. Because we did not observe evidence of better control of inflammation in the ICS group, this difference could be a direct effect of glucocorticosteroids on ASM contractility (51), possibly via the down-regulation of muscarinic receptors (52). However, as already mentioned, not all aspects of inflammation were investigated,

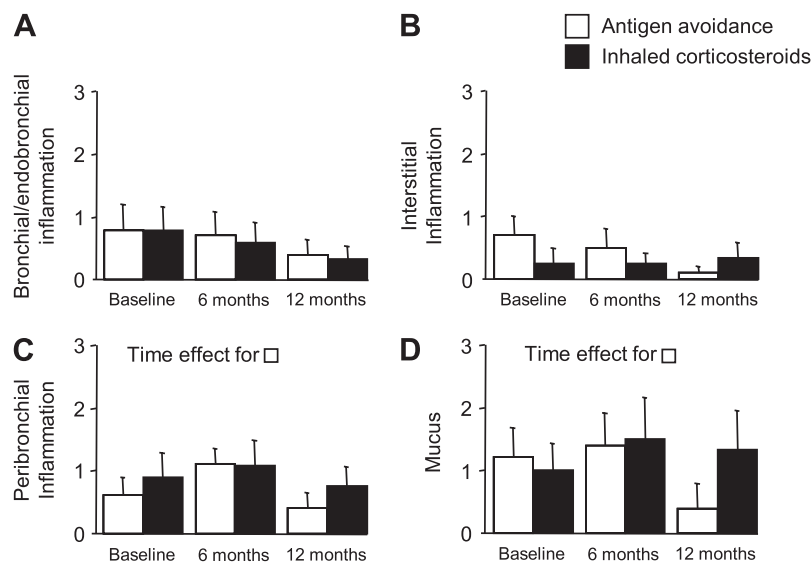


Figure 6. Histology of biopsies. Tissue inflammation and mucus accumulation in peripheral lung biopsies of heaves-affected horses after antigen exposure (Baseline) and after 6 and 12 months of treatment with antigen avoidance alone (open bars; $n = 5$) or inhaled corticosteroids (solid bars; $n = 6$). Inflammation (bronchial/endobronchial, A; interstitial, B; peribronchial, C) was scored from normal to marked on a scale of 0–3. Mucus was scored from 0–4. In the antigen avoidance group, an overall time effect ($P = 0.03$) was evident for peribronchial inflammation (C) and intraluminal mucus scores (D), with marginally lower scores at 12 months.

and the degree of activation of inflammatory cells could have influenced bronchoconstriction. Although persistent low-grade airway obstruction, despite long-term antigen avoidance, has been described (14), the residual reversible bronchoconstriction observed at 12 months was not expected. It raises the question of possible low-grade antigenic stimulation and residual inflammation despite strict environmental control and ICS treatment, because this persistent bronchoconstriction was similar in both groups.

Dissociation between Improvement in Airway Function and BAL Inflammation

The most striking dissociation between functional improvement and cytological evidence of inflammation was observed in the ICS group. Despite a positive effect of ICS on IL-8 expression, abnormal BAL cytology persisted as long as antigen exposure continued, and resolved when it ceased, indicating that the airway neutrophilia was not driven by ICS, but was uncontrolled by their use. This was not completely unexpected, because the effects of ICS on inflammation in heaves are inconsistent, even when lung function improves (53, 54). In a murine model of induced asthmatic inflammation, persistent BAL eosinophilia in dexamethasone-treated/antigen-exposed mice was also observed (55). This suggests that corticosteroids may not appropriately control airway inflammation when high antigen exposure is maintained. The modest effect on BAL inflammation could also be attributed in part to function-directed dosing. Higher doses may be required to control inflammation, and treatments based on inflammation markers would result in higher doses in people (56). A more subtle but perhaps more unexpected dissociation between inflammation and function was also seen in the antigen avoidance group. Although BAL neutrophilia had not changed at 1 month, resistance was significantly lower than at baseline, and elastance had already normalized. The heterogeneity of ventilation may have improved despite the persistence of inflammation because of a decrease of mucus accumulation. Overall, these observations support the notion that antigen avoidance and ICS are complementary, and that treatment efficacy should be monitored in terms not only of function but also of inflammation.

CONCLUSIONS

In this study, ICS accelerated the decrease in ASM remodeling when compared with antigen avoidance alone. However, this did not influence the total decrease in ASM observed during a 1-year period, and improvements remained modest. In addition, the data support a potential reversibility of subepithelial collagen remodeling with prolonged ICS or strict antigen avoidance. These results also suggest that part of ASM remodeling could be refractory to intervention. Nevertheless, we conclude that (1) an apparent benefit of ICS treatment is evident because of its more rapid effect on lung function and ASM remodeling, and (2) antigen avoidance leads to better control of inflammation, and it exerts additional beneficial effects when combined with ICS, even when clinical signs are well controlled by medication.

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Online data supplement

CORTICOSTEROIDS AND ANTIGEN AVOIDANCE DECREASE AIRWAY SMOOTH
MUSCLE MASS IN AN EQUINE ASTHMA MODEL

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Setlakwe, Guy Beauchamp, Christian Couture, James G. Martin, Jean-Pierre Lavoie

Methods

Experimental design and animals

All horses had a well-documented history of chronic heaves for more than 4 years, which was characterized by episodes of airway obstruction and inflammation upon hay exposure. Horses were deemed otherwise healthy based on physical examination, blood count and biochemistry. All procedures were performed in accordance with the Canadian Council for Animal Care guidelines.

Lung biopsies via thoracoscopy

Peripheral lung tissue of 8 to 12 cm³ was harvested in the caudo-dorsal region of the lung on standing, sedated animals (1). Samples were fixed for 24h in 4% formaldehyde and embedded in paraffin. At the end of the study, 3 horses in the ICS group and 2 in the antigen avoidance group were subjected to euthanasia for unrelated reasons (chronic lameness and limited housing), lung samples of the same size were collected in the same area immediately after euthanasia and analyzed similarly.

Immunostaining, enzymatic labeling and picrosirius-red staining

Immunohistochemical staining was performed for the co-localization of proliferating cell nuclear antigen (PCNA) with ASM on 5 µm sections using smooth muscle-specific α -actin mouse monoclonal antibody (clone 1A4, Sigma Immunochemicals, Toronto, ON), PCNA antibody (clone Ab-1, Calbiochem, San Diego, CA), and methyl green counterstaining (2, 3). Apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (ApopTag, Chemicon International, Temecula, CA), following the

manufacturer's instructions (3). The signal was developed with diaminobenzidine-nickel (Vector Laboratories, Burlington, ON), followed by eosin counterstaining. To ensure the specificity of the smooth muscle staining, double marking by immunofluorescence for smooth muscle α -actin (using the same antibody as for the immunohistochemical staining above) and smooth muscle-myosin heavy chain (rabbit anti-smooth muscle myosin II heavy chain, Biomedical technologies, BT-562) was performed and showed almost perfect overlap (Figure E1). Picrosirius-red staining was used to specifically stain collagen fibers, including thin filaments (4). A pre-digestion step with 0.5% papain solution was performed to remove proteoglycans and allow for a better penetration of the collagen fibers, followed by staining with 0.1% picrosirius-red as described elsewhere (5, 6).

Morphometry

In addition to airway smooth muscle (ASM) measurements described in the main manuscript, ASM nuclei were counted and corrected by the internal perimeter squared. ASM nuclei were also divided by the area of ASM measured, which is referred to as density of ASM nuclei (number of airways per animal/time point: 4 – 7, except for one with 3). To measure the subepithelial collagen area, i.e. the collagen present between the epithelium and the ASM layer, digitized airway images were transferred to the computer software program MBF Image J® (McMaster Biophotonics Facility Image J for Microscopy version 12.41, National Health Institute), with a threshold color function (Threshold color function Plugin, designed by G. Landini) to allow for specific subepithelial area collagen selection and its subtraction from the rest of the image. The area was corrected by the internal perimeter squared to account for variation in airway size (7). Epithelial detachment was measured as the percentage of the internal perimeter without epithelium adherent to the subepithelial extra cellular matrix.

Pulmonary function

Pulmonary resistance and elastance were measured as previously described (8). Briefly, pulmonary resistance and elastance are calculated from the flow rates obtained from a heated pneumotachograph attached to a mask and the transpulmonary pressure derived from an esophageal catheter, in unsedated animals. After the last function measurement at 12 months and to evaluate the presence of residual bronchoconstriction, a short-acting bronchodilator was administered (Salbutamol HFA 1000 µg, Ratiopharm Inc., Mississauga, Canada). Pulmonary function was measured 30 min later.

Bronchoalveolar lavage fluid collection

Two 250-mL boluses of isotonic saline were instilled in one main bronchus through a 1.6 m bronchoscope (Olympus Medical Systems Corp., Tokyo, Japan) as previously described (9). Bronchoalveolar lavage (BAL) fluid was transported on iced and processed within 2 hours of collection as described before (10). Briefly, BAL fluid was filtered, cells were washed twice in PBS, total cell count was determined with a hemocytometer, and cell viability was assessed by Trypan blue dye exclusion test. Aliquots of 10^7 cells were homogenized in TRIzol Reagent (Invitrogen, Burlington, ON, Canada) and immediately frozen at -80°C until analysis. Differential cell counts on Wright-Giemsa stained cytopins were obtained from a count of 400 cells (Hema 3, Fisher Scientific, Kalamazoo, MI). Additional slides were stained for mast cells using Toluidine Blue. The solution was prepared by dissolving 400 µl of acetic glacial acid and 0.025 g of Toluidine Blue in 30 ml of absolute ethanol, adding distilled water to make a total volume of 100 ml. Mast cell percentage was counted on these slides from a count 400 cells.

Bronchoalveolar lavage cell mRNA expression

RNA extraction was performed according to the manufacturer instructions (Invitrogen, Burlington, ON, Canada), as described elsewhere (10). RNA concentrations and 260/280 absorbance ratios (> 1.90) were evaluated by spectrophotometry (Nanodrop1000, ThermoScientific, Wilmington, DE, USA). Reverse transcription of 1 μ g total RNA was performed using Superscript III (500 U, Invitrogen) in the presence of RNasin Ribonuclease Inhibitor (60U, Promega, Fisher Scientific) and Oligo(dt) primers (500ng, Invitrogen) according to the manufacturer's instructions except that the reaction was performed in a final volume of 30 μ l. cDNA samples were stored at -20°C . There was one time point missing for one horse, and two time points for another one there, because of insufficient cell number.

Real-time PCR was performed with Quantitect SYBR Green PCR kit (Quantitect SYBR Green PCR kit, Qiagen, Mississauga, ON, Canada) according to the manufacturer's protocol and as described before (10), with minor modifications. Briefly, 2 μ l of cDNA was added to obtain 20 μ l final reaction volume containing 0.5 μ M of sense and antisense primers (primer sequences in Table 1). Cytokine primers were designed to span exon-intron boundaries in order to prevent amplification of genomic DNA. Amplification was performed on RotorGene thermal cycler (Rotor-Gene Real-Time Centrifugal DNA Amplification System 3000, Corbett Research, Montreal Biotech, Montreal, QC, Canada), with cycles optimized for each gene. The absolute concentration was obtained in cDNA samples using standard curves included in each run. Relative gene expression was calculated using the ratio of the target gene absolute concentration (ng/ μ l) divided by a reference gene appropriate for equine BAL, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (11). All samples were assessed in duplicate.

Histological scoring

For histopathological studies, lung tissue was stained with hematoxylin phloxine saffron stain. The histopathological scores were determined by a pathologist with experience in pulmonary pathology who was unaware of the status of the horses. A semi-quantitative scale was used to score tissue quality, inflammation (bronchial/endobronchial, peribronchial, interstitial) and intraluminal mucus accumulation. As a validation, a second evaluator also read the slides blindly and agreement was fair to excellent for all parameters.

Statistical analysis

Group characteristics were analyzed by Student's t-test (age and weight) and Fisher's exact test (proportion of mares). Bronchoalveolar lavage cell count, percentage of volume return, differential count, gene expression and epithelial detachment data were transformed (log transformed for cell count and gene expression, and arcsine square-root for volume, differential and epithelial detachment) prior to analysis. Correlation Z test between lung function and remodeling parameters (ASM and subepithelial collagen area) were done using all animals, at Baseline and 12 months. The software SAS 9.2 (SAS Institute, Cary, NC) was used.

Table 1. Primer pairs used for quantitative polymerase chain reaction

	Sense (5'→ 3')	Antisense (5'→ 3')
GAPDH	AAG TGG ATA TTG TCG CCA TCA AT	AAC TTG CCA TGG GTG GAA TC
IL-8	CTT TCT GCA GCT CTG TGT GAA G	GCA GAC CTC AGC TCC GTT GAC
TNF α	CTT GTG CCT CAG CCT CTT CTC CTT C	CAG CTG GTT GTC TGT CAG CTT C
TGF- β	CAG CAT GTG GAG CTG TAC CAG AAA	TGA CAT CAA AGG ACA GCC ATT CCG

GAPDH: glyceraldehyde 3-phosphate dehydrogenase, IL: interleukin, TNF: tumor necrosis factor, TGF: transforming growth factor.

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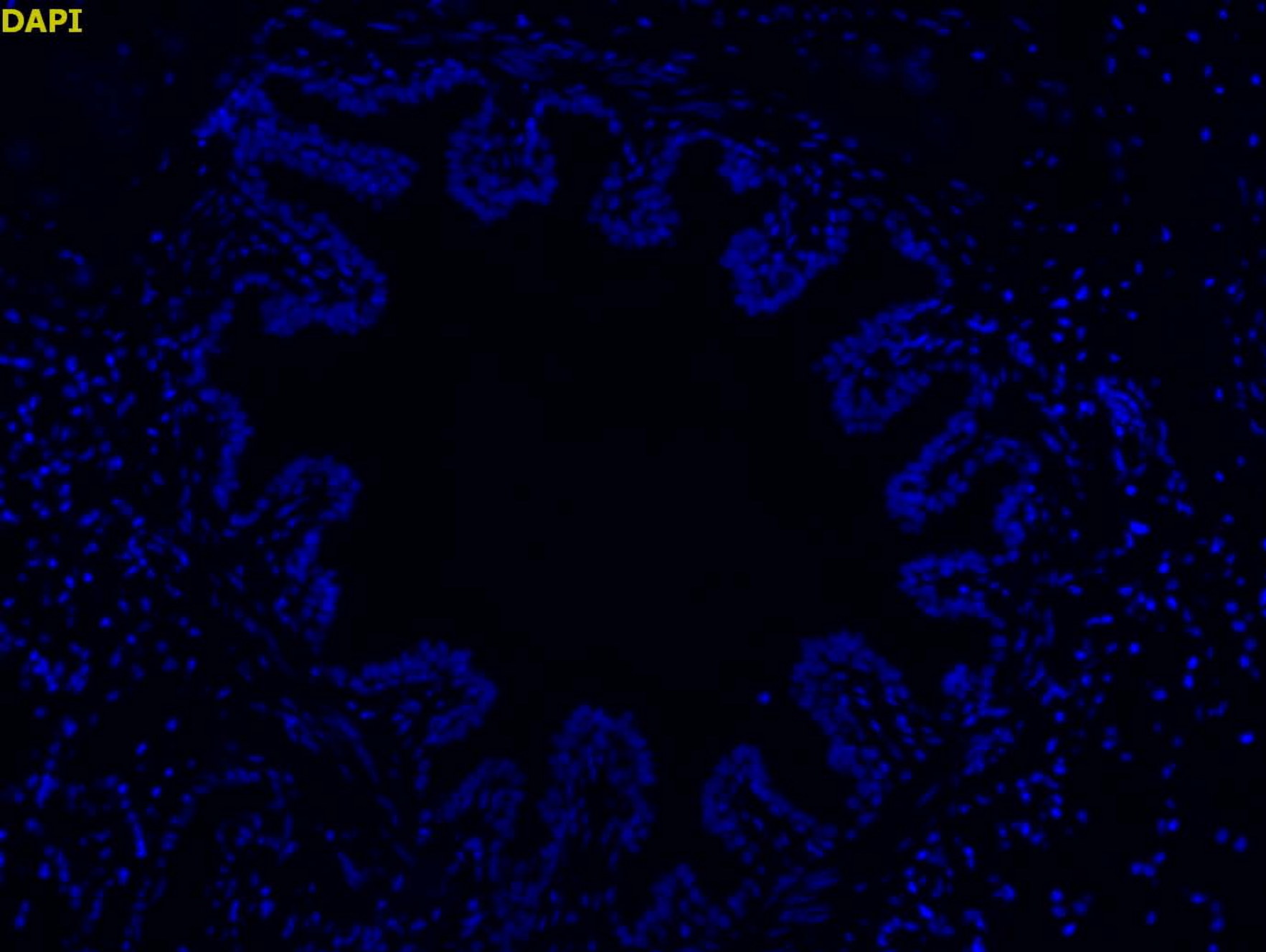
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Figure legend

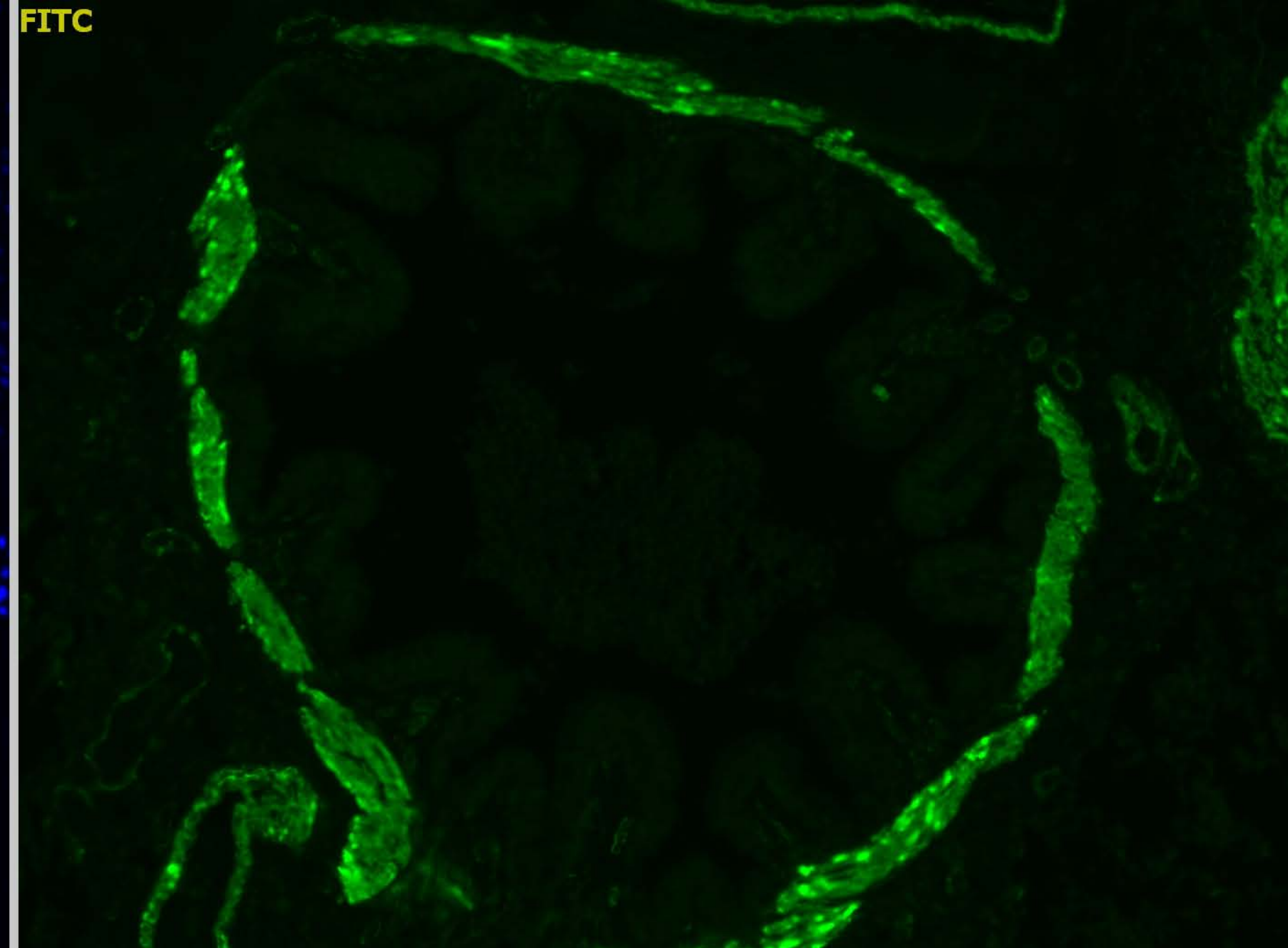
Figure E1. Double staining of peripheral airway smooth muscle of a heaves-affected horse.

Smooth muscle was double-stained using smooth muscle α -actin antibody (green) and smooth muscle-myosin heavy chain (red) and appropriate fluorescent-conjugated secondary antibodies. Nuclear staining in blue. Note that both antibodies appear to stain airway smooth muscle. Bar represents 100 μ m.

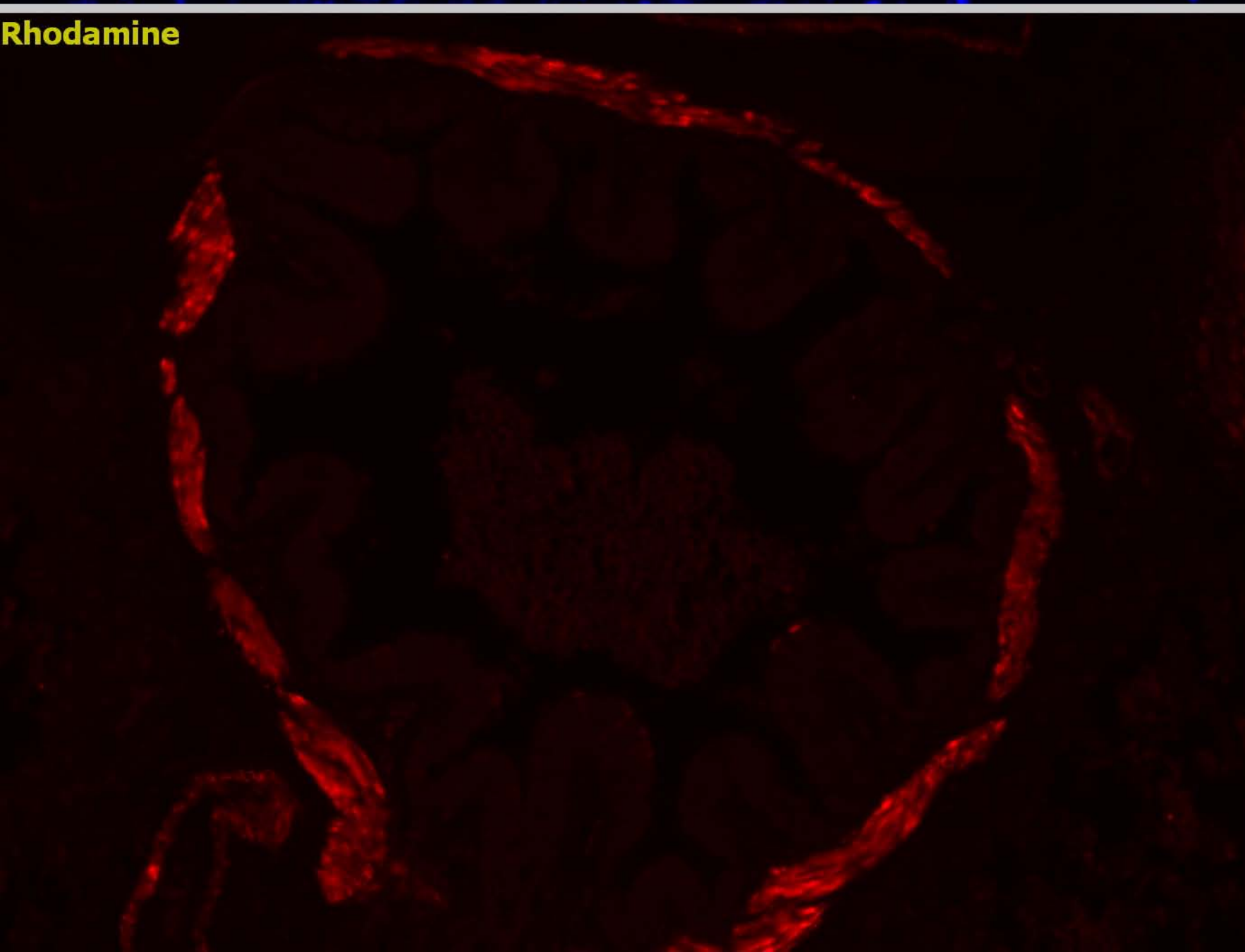
DAPI



FITC



Rhodamine



DAPI
+ FITC
+ Rhodamine

