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STANDARD ARTICLE

Efficacy of tamoxifen for the treatment of severe equine asthma

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Background: Tamoxifen, a selective estrogen receptor modulator, decreased airway neutrophilia and improved clinical signs in an experimental model of equine asthma, and induced neutrophilic apoptosis in vitro.

Hypothesis/Objectives: Tamoxifen reduces airway neutrophilia and improves lung function in severe asthmatic horses.

Animals: Twelve severe asthmatic horses from a research herd.

Methods: Randomized controlled blinded study design. The effects of a 12-day oral treatment with tamoxifen (0.22 mg/kg, q24h) or dexamethasone (0.06 mg/kg, q24h) on lung function, endoscopic tracheal mucus score and bronchoalveolar lavage fluid cytology were compared.

Results: Tamoxifen significantly improved the pulmonary resistance (R_L; mean reduction of 1.15 cm H_2O/L/s [CI: 0.29-2.01, P = .007] on day 13), but had no effect on the other variables evaluated. Dexamethasone normalized lung function (mean reduction of R_L of 2.48 cm H_2O/L/s [CI: 1.54-3.43, P < .0001] on day 13), without affecting airway neutrophilia.

Conclusions and Clinical Importance: Results of this study do not support the use of tamoxifen at the dose studied as an antineutrophilic medication in the treatment of asthmatic horses in chronic exacerbation.

KEYWORDS

airway neutrophilia, dexamethasone, estrogen, heaves

1 | INTRODUCTION

Severe equine asthma, also known as heaves or recurrent airway obstruction, is a common and incurable respiratory disease of adult horses. Exacerbations are triggered by inhalation of environmental antigens, most commonly those found in hay. The disease is characterized by airway hyper-responsiveness, mucus hypersecretion, intraluminal neutrophilia, and structural changes affecting the airways (remodeling).[1] Through the release of pro-inflammatory mediators, proteases, and extracellular traps, neutrophils are potentially major perpetuators of lung damage[2,3] and their presence has been associated with the dysfunction of peripheral airways in asthmatic patients.[4] Usual therapies (corticosteroids and bronchodilators), while improving the lung function, do not normalize airway luminal neutrophilia and tissue remodeling of asthmatic horses.[5,6] Furthermore, clinical signs relapse quickly after cessation of medication.[7] Although antigen avoidance controls airway inflammation, pulmonary remodeling is incompletely reversed even after a year at pasture.[5] Therefore, therapies targeting airway neutrophilia are required to determine if control of pulmonary inflammation can improve lung function and remodeling in severe equine asthma.[8]

Tamoxifen is a synthetic selective estrogen receptor modulator. Because of its antagonism of estrogen-dependent growth and its inhibitory effect on breast epithelial cells proliferation, its major use has been in the treatment of breast cancer.[9,10] Nonetheless, tamoxifen appears to have a broader spectrum of activity as it showed beneficial effects in estrogen-receptor negative cancers, in selected

Abbreviations: BALF, bronchoalveolar lavage fluid; CI, 95% confidence interval; E_L, pulmonary elastance; NETs, neutrophil extracellular traps; P_L, transpulmonary pressure; R_L, pulmonary resistance

immune disorders, and potentially in spinal cord injury.[11–14] As estrogen administration has been associated with both improvement and, contrariwise, to the development of asthma in women, studying the impact of an estrogen receptor modulator could help delineate the role of sex hormones in asthma.[15,16] Recently, it showed promising results for the treatment of severe equine asthma by reducing the neutrophilic chemotactic response and respiratory burst production and by inducing apoptosis of peripheral and pulmonary neutrophils in vitro.[17,18] Tamoxifen was also studied in healthy adult horses in which an asthma-like inflammation was experimentally reproduced by exposure to *Aspergillus fumigatus* contaminated hay. In this experiment, tamoxifen increased the apoptosis of peripheral and pulmonary neutrophils and improved clinical condition, airway neutrophilia, and mucus accumulation.[19] We therefore hypothesized that tamoxifen, by decreasing airway neutrophilia, would improve the lung function of severely asthmatic horses. These objectives were to study the effects of tamoxifen on airway luminal inflammation, on tracheal mucus accumulation and on pulmonary function testing of asthmatic horses during continuous antigen exposure.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experimental procedures were performed in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal (Protocol # Rech-1324).

2.2 | Animals

Twelve severe asthmatic horses (7 mares and 5 geldings) from this research herd were studied. Horses were mixed breeds, aged 14 ± 4 years and weighed 514 ± 51 kg. The horses were diagnosed with severe asthma based on history and previous results of pulmonary function and bronchoalveolar lavage fluid (BALF) cytology. These horses had historically >25% neutrophils on BALF cytology and a transpulmonary pressure (P_L) change above 15 cm of H_2O when stabled and fed hay and airway obstruction was reversible by antigen avoidance. The presence of a concomitant medical condition was excluded with a physical examination and complete blood count.

To induce chronic exacerbation of the disease as seen in clinical practice, the animals were stabled 3 weeks before the study and were fed dry timothy hay and sweet feed twice daily. The management remained the same throughout the study period. No treatment was administered at least 7 weeks before the trial. Horses were conditioned to wear a mask and to stand in a stock.

2.3 | Pulmonary function tests

Lung function was measured in standing unsedated animals, except for one horse that required sedation before each pulmonary function test (xylazine [Rompum, Bayer, Mississauga, ON, Canada], 0.4 mg/kg, IV).[6] Briefly, esophageal pressure was measured as an index of the transpulmonary pressure (P_L) with a balloon sealed over the end of a polyethylene catheter placed in the distal third of the esophagus. Flow rates were obtained by the use of a heated pneumotachograph and a differential pressure transducer fitted to a mask placed over the horse's nose. The system (Flexiware 7.6, SCIREQ, Montréal, QC, Canada) allowed electronic integration of the flow signal to provide tidal volume. Before each experiment, the system was calibrated by forcing known flow of air through the pneumotachograph with a blower-rotameter and by applying known pressure with a water manometer on the differential pressure transducer used to measure esophageal pressure. Values of pulmonary resistance (R_L) and elastance (E_L) were obtained by applying the data to the multiple regression equation for the single compartment model of the lung $(P_L = E_L V + R_L V + K)$ where V is the volume, V the airflow, and K the transpulmonary end-expiratory pressure. All the valid breaths were used for analysis.

2.4 | Endoscopic tracheal mucus scoring and bronchoalveolar lavage

Horses were sedated with xylazine (Rompum, Bayer, Mississauga, ON, Canada; 0.5 mg/kg, IV) and butorphanol (Torbugesic, Zoetis, Florham Park, New Jersey); 20-30 μg/kg, IV) and tracheoscopy was performed with a 1.6 m videoendoscope (Evis Exera II CV-180, Olympus Canada Inc., Richmond Hill, ON, Canada). Tracheal mucus score was evaluated during reviewing of video recordings by an investigator blinded to the treatment group.[20] Bronchoalveolar lavage was performed as previously described.[6] Briefly, after topical anesthesia with 0.5% lidocaine (Lurocaine; lidocaine hydrochloride 20 mg/mL, Vétoquinol N.-A. Inc., , Lavaltrie, QC, Canada), two 250 mL-boluses of warm sterile isotonic saline (0.9% Sodium Chloride Injection, USP, Baxter, Mississauga, ON, Canada) were sequentially instilled into a main bronchus through the videoendoscope and then aspirated with a suction pump. The samples were kept on ice until reaching the laboratory within 90 minutes. Cytocentrifuged preparations of BALF (400 μL, unfiltered) were made and cells were stained with a modified Wright–Giemsa solution (DiffQuick, Fisher Scientific, Waltham, Massachusetts). Differential leucocyte counts from 400 cells were performed by an investigator blinded to the treatment group.

2.5 | Study protocol

After randomization based on pulmonary resistance ranking value, six horses received tamoxifen citrate (Apo-tamox, Apotex Inc., Toronto, ON, Canada); 20 mg/tablet, 0.22 mg/kg, PO)[19] once daily and six horses received dexamethasone[i] (Dexamethasone powder, Dominion Veterinary Laboratories Ltd. Winnipeg, MB, Canada); 10 mg/packet, 0.06 mg/kg, PO) once daily for 12 days. Pulmonary function tests were performed before treatment on day 1, and on days 6 and 13. Endoscopic tracheal mucus scores and bronchoalveolar lavages were performed on days 1 and 13. The attitude, appetite, and a clinical respiratory score[21] were evaluated daily by a blinded investigator.

2.6 | Statistical analysis

Bronchoalveolar lavage, lung function, and the clinical respiratory score data were analyzed with repeated-measures two-way ANOVA with "group" as the between-subject factor and "time" (days of

treatment) as the within-subject factor with Bonferroni corrections for multiple comparisons. Mucus scores were evaluated within each treatment group with Wilcoxon matched-pairs signed rank tests and among treatment groups with Mann-Whitney *U* tests. Data are described as mean difference with 95% confidence interval (CI). *P* values <.05 were considered statistically significant. GraphPad Prism 7 (GraphPad Prism 7, GraphPad Software, Inc, La Jolla, California) was used for statistical calculations.

3 | RESULTS

No adverse effect was observed in the tamoxifen group. On the ninth day of treatment, a horse treated with dexamethasone developed hypocalcemic and hypomagnesemic tetany unresponsive to treatment. Euthanasia was humanely elected for this animal and at necropsy, loss of the principal cells of the parathyroid gland was observed. The exact cause of this finding was undetermined. All data from that horse were excluded from analysis.

3.1 | Pulmonary function tests and clinical respiratory scores

Horses were in clinical exacerbation of the disease at baseline (P_L > 15 cm H_2O, R_L > 1 cm H_2O/L/s, and E_L > 1 cm H_2O/L). A group-time interaction was noted for the R_L, E_L, and P_L values as dexamethasone significantly improved lung function which normalized in all treated horses when evaluated on day 6 (mean reduction of R_L of 2.29 cm H_2O/L/s [CI: 1.35-3.24, *P* < .0001], mean reduction of E_L of 4.44 cm H_2O/L [CI: 2.51-6.37, *P* < .0001], and mean reduction of P_L of 32.44 cm H_2O [CI: 20.69-44.20, *P* < 0.0001]) and 13 (mean reduction of R_L of 2.48 cm H_2O/L/s [CI: 1.54-3.43, *P* < .0001], mean reduction of E_L of 4.45 cm H_2O/L [CI: 2.52-6.38, *P* < .0001], and mean reduction of P_L of 34.65 cm H_2O [CI: 22.89-46.41, *P* < .0001]; Figures 1–3). Treatment with tamoxifen had no significant effect on P_L (mean increase of 2.19 cm H_2O on day 13 [CI: −12.93 to 8.54, *P* > .99]) and E_L (mean increase of 1.74 cm H_2O/L on day 13 [CI: −3.51 to 0.02, *P* = .054]). However, a significant reduction of R_L was noted at the end of the treatment period with tamoxifen (mean reduction of 1.15 cm H_2O/L/s [CI: 0.29-2.01, *P* = .007]). The clinical respiratory scores were improved

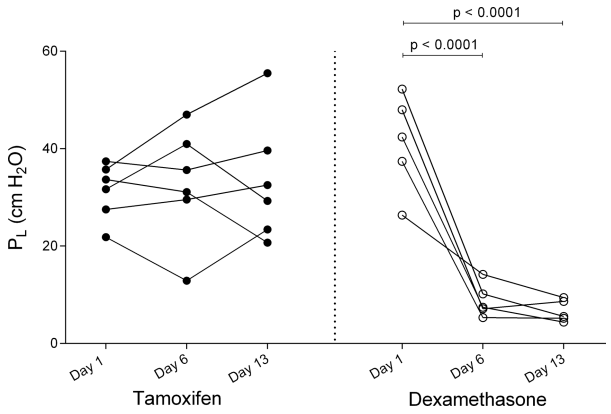

FIGURE 1 Values of transpulmonary pressure (P_L) on day 1 (before administration of medication), day 6 and day 13 of treatment with tamoxifen (black circles) and dexamethasone (white circles)

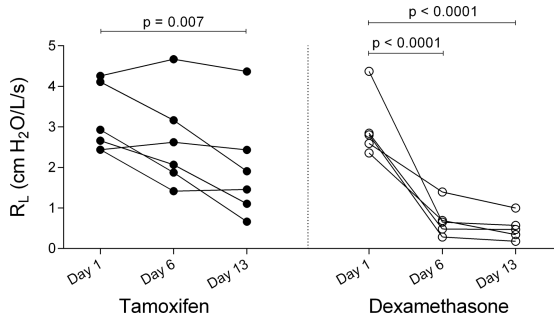

FIGURE 2 Values of pulmonary resistance (R_L) on day 1 (before administration of medication), day 6 and day 13 of treatment with tamoxifen (black circles) and dexamethasone (white circles)

in the dexamethasone group only, on day 6 (mean reduction of 1.6/8 [CI: 0.2-3, *P* = .01]), 8 (mean reduction of 1.8/8 [CI: 0.4-3.2, *P* = .004]), 10 (mean reduction of 1.8/8 [CI: 0.4-3.2, *P* = .004]), and 11 (mean reduction of 2.2/8 [CI: 0.8-3.6, *P* = .0002]; Figure 4).

3.2 | Tracheal mucus scores

No difference was observed in the tracheal mucus scores among treatment groups at baseline or after treatment (mean reduction of 0.8/5 [CI: −2.8 to 1.1, *P* = .56] in the tamoxifen group and mean reduction of 0.5/5 [CI: −2.0 to 1.0, *P* = .75) in the dexamethasone group on day 13; Figure 5).

3.3 | BALF cytology

At the beginning of the study, the percentage of neutrophils in the BALF was above normal (>5%) in 10 of the 11 remaining horses. The horse lacking airway neutrophilia was excluded from analysis concerning BALF cytology. The two-way ANOVA showed a group effect (*P* = .02) which was possibly related to a higher neutrophilia at baseline in the tamoxifen group. Neither tamoxifen (mean reduction of 17.3% [CI: −15.1 to 49.8, *P* = .35]) nor dexamethasone (mean reduction of 3.3% [CI: −29.2 to 35.7, *P* > .99]) improved the BALF neutrophilia (Figure 6).

4 | DISCUSSION

The results of this randomized controlled study failed to detect an effect of a short-term treatment with tamoxifen on airway neutrophilia in

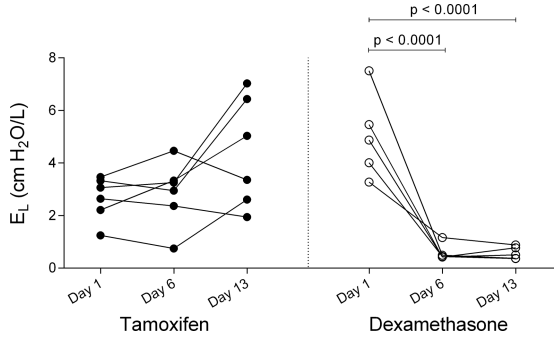

FIGURE 3 Values of pulmonary elastance (E_L) on day 1 (before administration of medication), day 6 and day 13 of treatment with tamoxifen (black circles) and dexamethasone (white circles)

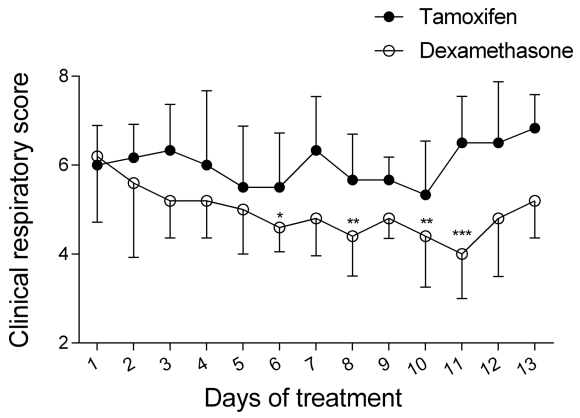

FIGURE 4 Daily clinical respiratory scores (means and standard deviations) from day 1 (before administration of medication) to day 13 of treatment with tamoxifen (black circles) and dexamethasone (white circles). *P < .05; **P < .01; ***P < .001 (significant differences from baseline)

severe asthmatic horses, refuting this hypothesis. A statistically significant diminution, but not a normalization, of airway resistance was observed, without improvement of the pulmonary elastance. As expected, dexamethasone normalized lung function while neither reducing airway inflammation[6] nor macroscopic mucus accumulation [22].

Tamoxifen's effectiveness in the treatment of breast cancer is partly attributed to the apoptosis of tumor cells mediated by oxidative stress and increased ceramide intracellular level among other mechanisms.[23] Tamoxifen also causes ceramide intracellular accumulation in neutrophils[24], therefore, we suspected that it could lead to neutrophilic apoptosis and concurrent improvement of airway inflammation. However, we did not observe a decrease of airway neutrophilia with tamoxifen which contrasts with previous reports. Indeed, in one study, tamoxifen administered every other day for three doses reduced experimentally induced intraluminal inflammation in healthy horses.[19] However, the pathways responsible for neutrophil accumulation in healthy horses after hay exposure likely differ from that of asthmatic horses which might explain these different results. In addition, because the airway neutrophilia is transient when hay is introduced to healthy horses and considering that antigenic exposure was ceased when the treatment was initiated, the improvement reported might have been the normal kinetic of airway inflammation regulation, rather

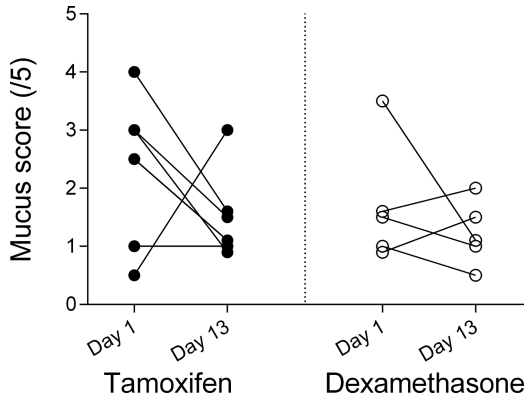

FIGURE 5 Tracheal mucus scores on day 1 (before administration of medication) and day 13 of treatment with tamoxifen (black circles) and dexamethasone (white circles)

than an effect of tamoxifen.[25] Moreover, the duration of the antigenic challenge was of a short duration (1 week) when compared with this study (3 weeks), which could have contributed the discordance of the results. The chronicity of the exacerbation in this study could have impeded the anti-inflammatory efficacy of this medication. However, to be useful clinically, a treatment would have to be effective under these conditions.

The effects of tamoxifen on neutrophils are controversial with in vitro studies suggesting an anti-inflammatory effect by the induction of apoptosis, a reduction of the chemotactic response and respiratory burst production, a decreased production of 5-lipoxygenase and a diminution of the neutrophilic infiltration to the site of injury.[17–19,26,27] In contrast, other experiments have shown activation of neutrophils by an enhancement of their chemotaxis, phagocytic and bactericidal activity, and neutrophil extracellular traps (NETs) formation.[24] Because aberrant NETs production is a feature of human and equine asthma, medications increasing their formation might be detrimental for the treatment of this condition.[3,28] Furthermore, it has been suggested that the immunomodulation associated with tamoxifen is mediated by a shift from a Th1 to a Th2 response, possibly related to an inhibition of the maturation of dendritic cells.[11,29] Because a predominant Th2-type response has been associated with exacerbation in severe equine asthma, at least in some horses [30,31], a shift in cellular signaling might explain the lack of efficacy in this study. Of note, a case report describes human asthma exacerbations induced by tamoxifen, but the mechanisms of those deteriorations were not determined.[32] Taken together, the usefulness of this medication raises interrogations in a disease where immunological pathways involved are complex and incompletely understood.

The main limitation of this trial is the small number of horses which might have precluded the detection of small differences in the airway neutrophilia, the primary outcome of this study. In addition, the mild neutrophilia (<25%) observed in five horses in the present trial could have reduced our capacity to identify improvement of luminal inflammation. Even though the BALF cytology is a mainstay in asthma diagnosis, the degree and onset of neutrophilic influx might not be constant.[33] From these five horses, only one had airway neutrophilia defined as normal (<5%) despite its disease being well characterized by being part of the research herd for several years, and the presence of severe airway obstruction (R_L of 4.3 cm H_2O/L/s and E_L of 2.2 cm H_2O/L) at the onset of the study. Nevertheless, the changes in airway neutrophilia with tamoxifen were inconsistent, and did not lead to normalization in any horses. The sample size was based on power analysis calculated from the Perez's study[19] results where six animals in each group was sufficient to observe significant improvement in airway inflammation, clinical score, and mucus accumulation with tamoxifen. However, considering the mild reduction of neutrophilia and the variability of the data in the current study, about 30 severe asthmatic animals would have been required to obtain significant result with a power of 80% and alpha set at 0.05. Such a small effect would question the usefulness of this drug for the treatment of severe equine asthma. Finally, we cannot exclude that the lack of improvement because of inappropriate dosage. The dose was chosen because it was reportedly effective at decreasing the airway neutrophilia in experimental airway inflammation in horses and was similar

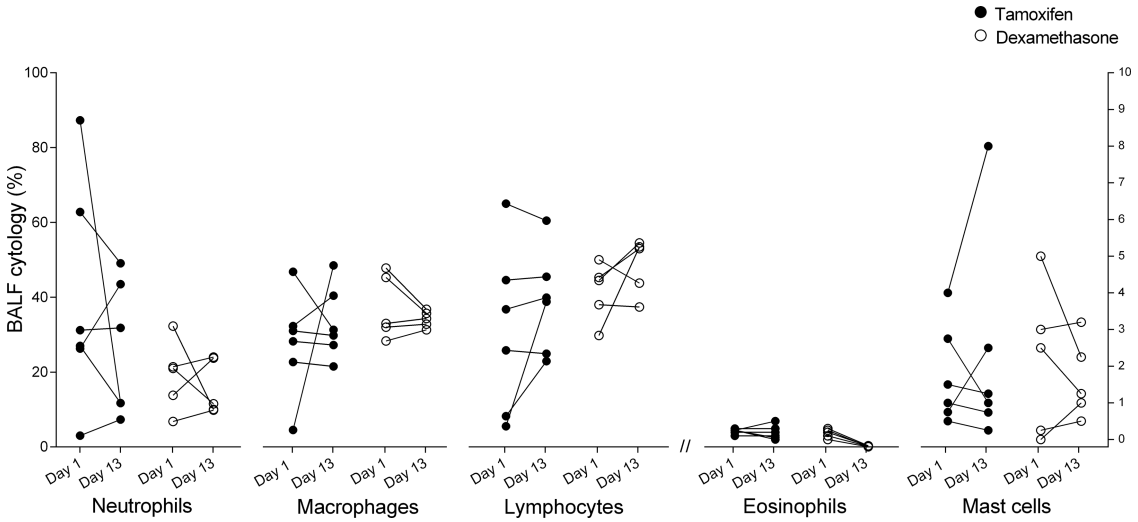

FIGURE 6 Percentage of each leucocyte population in the bronchoalveolar lavage fluid (BALF) before (day 1) and after treatment (day 13) with tamoxifen (black circles) and dexamethasone (white circles). The left *Y* axis applies to the percentage of neutrophils, macrophages and lymphocytes. The right *Y* axis applies to the percentage of eosinophils and mast cells

to the dosage used for breast-cancer treatment.[10,19] The pharmacokinetics of the molecule is currently studied (G. Morán, personal communication). The results of this study should be interpreted in light of the limitations raised above.

The clinical significance of the improvement in R_L with tamoxifen in the present study is difficult to conceptualize because the other parameters of lung function and the clinical respiratory scores were unchanged. Resistance is associated with airflow limitations in central airways. Therefore, it is possible that tamoxifen-mediated specific bronchodilator effect on central bronchi independently from an effect on airway luminal neutrophils and without improving airflow in the periphery of the lung. Selective estrogen receptor modulators can act as a receptor antagonist or agonist depending on the target cells. Interestingly, estrogen possesses bronchodilator property which might be related to its receptor colocalization with beta-2 adrenergic receptors in airway smooth muscle cells.[34] Some studies associate estrogen with improvement of asthma clinical signs or lung function[15], whereas others suggest a detrimental effect of this sex hormone[16]. Therefore, an estrogen-mediated bronchodilation would be a possible explanation for the reduced R_L observed in this study, however more research on the role of sex hormones in asthma is required. Alternatively, the bronchodilation could have been mediated by an interaction with calcium-channels. A reduction of vascular smooth muscle cell contractility has been reported with tamoxifen and the authors suggested that the effect might be related to an inhibition of voltage-dependant calcium channels.[35]

Consistent with the favorable safety profile of tamoxifen in humans, we did not observe adverse events with a short-term administration. Reported adverse effects after prolonged use include endometrial cancers and thromboembolic accidents.[9] The refractory hypocalcemic and hypomagnesemic tetany observed in a dexamethasone treated horse was associated with the loss of principal cells in the parathyroid gland which possibly decreased the production of parathyroid hormone. Because glucocorticoids alter calcium metabolism by increasing urinary excretion and decreasing intestinal absorption, the primary underlying condition might have been worsened by the dexamethasone treatment.[36,37]

In conclusion, a short-term tamoxifen treatment failed to improve airway inflammation of severely asthmatic horses in chronic exacerbation, precluding the use of this medication at the current posology to assess the role of pulmonary neutrophils in the disease. The treatment resulted in a reduction of R_L, but in contrast to dexamethasone, the lung function did not normalize. Nevertheless, deciphering the mechanisms responsible for the improvement in lung function of severe asthmatic horses with tamoxifen is of interest and justifies determining its pharmacokinetics for future studies.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All experimental procedures were performed in accordance with the Canadian Council for Animal Care guidelines and were approved by

the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal (Protocol # Rech-1324).

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