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# SERUM CORTISOL AS A SYSTEMIC MARKER IN THE LONG-TERM TREATMENT WITH FLUTICASONE PROPIONATE (FP) IN RECURRENT AIRWAYS OBSTRUCTION (RAO) AGED HORSES

## Cortisol sérico como un marcador sistémico en el tratamiento prolongado con propionato de fluticasona en caballos viejos con obstrucción recurrente de las vías aéreas

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ABSTRACT

This is the first fluticasone propianato (FP) long-term study in Recurrent Airways Obstruction (RAO)-aged horses (*Equus ferus caballus*) that compare Fluticasone Propionate (FP) treatment regimens, as well as FP treatment vs. environmental control, over Hypothalamus Pituitary adrenal (HPA) axis, its reversibility and cortisol suppression clinical consequences. The goal was to follow-up RAO clinical signs and cortisol levels in aged RAO-horses treated with FP. Eleven horses were stabled in moldy-dusty conditions to cause RAO disease. Once they were RAO symptomatic, they were divided in two groups: RAO-FP (treatment group) that were kept over hay/straw and RAO-control (non-treatment) that were kept over pellets/wood shavings by six months. After that, all the horses were kept on the paddock and pasture. FP (Flovant HFA®) 2000µg B.I.D.(twice-a-day) during six months and 2000µg S.I.D. (once-a-day) during other five months was given using Aeromask®. Clinical respiratory signs (CRS) were also recorded (nasal flaring and abdominal movement). Cortisol was measured using commercial chemiluminescence (CEIA) kit (Immulite 1000®, Siemens) -12, -10 d before and 7, 28, 80,160,200,250,290, 320 d after treatment. Cortisol was used as systemic biomarker in determining any potential suppression and general effect related to FP treatment. FP 2000µg B.I.D. was associated with a decrease in blood cortisol levels on 28, 80 and 160 d in RAO-horses, however cortisol levels returns similar to baseline values, as well as similar to cortisol values in RAO-control horses when same FP dose was given just S.I.D. These results suggest that long-term 2000µg/horse B.I.D. inhaled FP treatment suppresses endogenous cortisol through its action over the hypothalamic–pituitary–adrenal (HPA) axis. Cortisol suppression with FP 2000 µg/horse B.I.D., may have deleterious effects if suppression is maintained for more than six months in those aged horses,proving that FP dose should be adjusted and combined with environmental management, according to clinical signs improvement.

**Keywords:** RAO, cortisol, fluticasone, HPA dysregulation, heaves.

RESUMEN

Este es el primer estudio clínico controlado en caballos viejos (*Equus ferus caballus*) con asma (RAO) que compara diferentes regímenes de tratamiento de propionato de fluticasona (FP), así como también el tratamiento de FP versus el control del ambiente, sobre el eje hipotálamo-hipófisis-adrenal (HHA), su reversibilidad y las consecuencias clínicas de la supresión. El objetivo fue hacer un seguimiento de los signos clínicos del asma y niveles de cortisol de los caballos tratados con FP. Los caballos fueron estabulados en un ambiente con condiciones mohosas para causar el asma, una vez que los caballos fueron sintomáticos, fueron divididos en dos grupos: RAO-FP (grupo asma con tratamiento) que fueron mantenidos en heno/paja y un grupo RAO-control (grupo asma sin tratamiento) que fue mantenido sobre alfalfa peletizada/viruta de madera por seis meses. Después de este periodo, todos los caballos fueron puestos en el corral y potrero. FP (Flovant HFA®)

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2000µg B.I.D. (dos veces al día (d)) durante seis meses y 2000µg S.I.D. (una vez al día) durante otros cinco meses, fue administrado usando Aeromask®. Los signos clínicos respiratorios (CRS) fueron también registrados (presencia o no de respiración abdominal y dilatación o no de orificios nasales). Cortisol fue medido usando un kit comercial de quimioluminiscencia (CEIA) (Inmulite 1000®, Siemmens. Canada) -12,-10 d antes y 7; 28; 80; 160; 200; 250; 290 y 320 d después de empezado el tratamiento. El cortisol fue usado como un biomarcador para determinar cualquier potencial de supresión y efecto sistémico relacionado con el FP. FP 2000 µg dos veces al d fue asociado con una reducción de los niveles de cortisol los d 28; 80 y 160, en caballos con obstrucción recurrente de las vías aéreas tratados con FP; sin embargo, los niveles de cortisol retornaron a los valores normales de base, como también a valores similares a los valores de cortisol de los caballos control cuando el FP fue administrado solo una vez al d. Estos resultados sugieren que 2000 µg de FP B.I.D. inhalado/caballo suprime el cortisol endógeno por su efecto en el eje HHA. La supresión del cortisol con 2000 µg/caballo B.I.D. puede tener efectos deletéreossi la supresión sobre el cortisol es mantenida por más de seis meses en estos caballos viejos, probando que la dosis de FP debe ser ajustada y combinada con manejo ambiental de acuerdo al mejoramiento de los signos clínicos.

**Palabras clave:** RAO, cortisol, fluticasona, HHA, asma equino.

## INTRODUCTION

Recurrent Airways Obstruction (RAO) is a chronic airway inflammatory disease common in horses (*Equus ferus caballus*) confined instable for long winter periods, and exposed to moldy-dusty hay and straw. The condition may be controlled by the use of bronchodilators and steroids as well as environmental management in order to reduce the animal exposure to dust and other allergens [32, 36, 54, 71]. RAO horses usually show nasal secretions and concurrent cough, especially during exercise and feeding time, as well as exercise intolerance [38]. The frequency and severity of coughing increases as the disease progresses and in some cases the horses have paroxistical nonproductive cough; develop severe respiratory distress with increased respiratory rate and effort, showed by flared nostril, extended neck and head, coughing at rest, and double expiratory effort that lead to external abdominal hypertrophy [36, 39, 54]. RAO is a multi-factorial disease that could have a genetic predisposition [52], nonetheless environment has also been implicated. Horses older than four years of age are several times more prone to develop RAO symptoms [15] Although RAO has been mainly described in template countries and aged horses, a study done in Venezuela under warm tropical conditions have found 69% RAO prevalence, among 2-6 years old Thoroughbred horses, stabled and racing [66].

Most of the RAO horses in the acute phase are so clinical evident because of their respiratory effort, as well as increase of pleural pressure (delta max Ppl) and pulmonary resistance (RL), and dynamic compliance (Cdyn) during lung function test [13, 59]. A strong correlation has been established between severity pulmonary disease, clinical respiratory score and pulmonary function [25, 57, 60].

In chronic airways inflammation pathologies like asthma, tissue damage and structural changes are labelled as airway remodelling [69, 70]. These structural changes range from epithelial goblet cell hyperplasia, mucous gland hyperplasia-metaplasia [2] reticular basement membrane thickening and increased vascularity of mucosa with thickening of smooth muscle layer [30, 51, 69]. RAO airway remodeling ranges from epithelial goblet cell hyperplasia, mucous gland hyperplasia-metaplasia, reticular basement membrane thickening and increased vascularity of mucosa with thickening of smooth muscle layer [38, 69].

Current asthma therapies are mostly effective in ameliorating inflammatory responses if airway remodeling processes are not already in progress, once they are present the animal became poorly responsive to those agents [47, 69]. Therefore, new therapeutic alternatives and approaches are required to control and prevent asthma as well the RAO symptoms in horses [33]. One option to improve clinical signs is controlling the environment in order to reduce allergens exposure [9, 10, 37] through the use of pelleted feeding [39] and improving air breathing quality by promoting good ventilation. Both management strategies help to decrease ammonia gases and improve air quality, as well as bacteria suspension in dust [9, 10, 44, 50]; but for horses that remain indoor all year round, additional therapeutic practices with systemic steroids (e.g. dexamethasone, prednisolone, isoflupredone, triamcinolone) or inhale administration (e.g. beclomethasone, fluticasone) could be required as an alternative to relief the clinical signs of airways obstruction [14, 34, 35, 49, 59, 60, 61, 62].

Glucocorticoids are crucial anti-inflammatory drugs that play a key role in the treatment of local and systemic inflammatory pathologies and diseases. Clear knowledge of the pharmacokinetics and pharmacodynamics of the different available glucocorticoids is necessary to assess their efficacy and safety profile. Inhaled corticosteroids are mainly used as first-line treatment for asthmatic patients and RAO horses because they exert a local effect at the site of action and thus decrease the risk for adverse drug reactions of systemic corticosteroids [7, 13, 22].

In equine practice, Fluticasona Propionate (FP) is one of the most commonly used inhaled steroids. FP is recommended for controlling RAO and inflammatory airway disease symptoms in horses. In RAO-affected horses, FP (2000 µg B.I.D. for three weeks) improves lung function; however comparable results have been seen in only two weeks when the drug is combined with environmental management practices [14].

Synthetic (exogenous) or endogenous glucocorticoids are capable to inhibit corticotropin-releasing factors, stress induced adrenocorticotropic hormone (ACTH) and arginine-vasopressin secretion [68].Corticosteroid feedback inhibits the brain-hypothalamus-pituitary units of the adrenocortical system. Endogenous corticosteroids may have their primary actions at brain and hypothalamic sites and synthetic glucocorticoids that do not bind to transcortin may act primarily on corticotropes and regions of brain outside the blood–brain barrier [27-29]. This inhibition at the level of the pituitary and hypothalamus is mediated through a negative feedback mechanism that controls secretion and synthesis [27-29]. Others have suggested direct inhibition of the cortisol secretions by the adrenal cortex, without the involvement of ACTH and the central hypothalamic-pituitary axis (HPA) [28, 29].

Glucocorticoids also regulate cytokines and inflammatory mediators'synthesis by establishing a negative feedback loop on the brain inhibiting the HPA axis [28, 29] Dysregulation of this balanced neuroendocrine feedback by hyperactivity (e.g. Cushing's disease, pain, caloric restriction, emotional trauma or exogenous corticosteroids) or hyporeactivity (e.g. Addison's disease) of the HPA axis causes systemic alterations in inflammation and immunity [22, 41, 53, 64, 73].

HPA dysregulation also occurs in patient with acute inflammation pathologies as acute respiratory distress syndrome [41] that may also occurs in RAO in horses. On the other hand, HPA hyperactivity, as in Cushing's syndrome, without an underlying inflammation etiology may cause immunosuppression and susceptibility to infections [42, 73].

This study aim to describe the effect of two therapeutic schedules of a long term FP treatment on the cortisol suppression and its reversibility, by assessing plasma cortisol levels in aged horse with RAO.

## MATERIALS AND METHODS

### Animals

Eleven (11) RAO horses mixed breed horses (7 mares and 4 geldings) aged 18–20 years (mean +/- SD, 19.9 +/- 2.1 years), weighing 419– 550 kg (465+/-35 kg) were used in this study. Those horses, after a controlled exposure to hay, were confirmed suffering RAO according to history and symptoms, then they were graded using a clinical score [54, 60, 63]. Horses in this research belonged and were part of a larger study evaluating the reversibility of pulmonary remodeling from the Respiratory Cellular and Molecular Biology Laboratory of the Université de Montreal [16, 37]. Basic veterinary care was routinely performed, and all the horses were dewormed and vaccinated annually against (tetanus, West Nile, Eastern and Western equine encephalitis, equine herpes virus 1/4, influenza, and rabies vaccines). Horses were stabled in research barn facilities and only moved to hospital facilities for close monitoring on thoracoscopy surgical time.

Horses were kept in door under controlled humidity (60-70%) and temperature (14-17°C) conditions for two months before the study was initiated and during six months after RAO clinical signs started. In the first stage of the study (antigenic challenge-T0) horses were fed with bad quality hay-straw bedding and on regular basis, and were exposed to dust by shaking dusty-hay inside boxes, in order to exacerbate RAO symptoms. During the second stage (T1), horses were randomly divided in two groups: RAO-Control (fed with pelleted alfalfa and kept on wood shaving bedding) and RAO-FP treatment group (fed with moldy-hay and kept on straw bedding). Finally, in the last stage of the study (T2), all horses were released to paddock and maintained on pasture. Horse were kept indoors only at night and always sampled before releasing.

Horses were under veterinary supervision on daily basis. Before blood sampling for cortisol, CBC and biochemistry, a systematic physical examination (temperature, respiratory and cardiac frequency) was done routinely to rule out any infection or disease, which could potentially, alter cortisol values by non-drug or non-experimental related reasons. All experimental procedures (such as treatment application, handling and sampling of animals) were done at Université de Montreal Research Facilities (located in St. Hyacinthe, Quebec. Canada) in compliance with the Canadian Council of Animal Care and approved by the Université de Montreal Animal Care Committee.

### Experimental design

This case-control clinical study was designed to make an assessment of cortisol in RAO horses treated with Fluticasone propionate (FP) compared to RAO-control horses. The designed was as follow, all horses were stabled in dusty-hay environment and exhibit RAO symptoms, this was considered time zero (T0). RAO-horses were divided in two groups: one group treated with fluticasone propionate (RAO-FP), fed with moldy-hay and kept on straw bedding and another group without FP (RAO-control), fed with pelleted alfalfa (*Medicago sativa*) and kept on wood shaving bedding. RAO-FP group received 2000 µg of inhaled FP B.I.D. for six months (T1). FP was administered using a metered-dose inhaler (8 puffs of 250 µg Flovent HFA®) and commercially delivering system, specially designed for horses (aeromask® and aero-chamber®). After six months FP was reduced to 2000 µg of FP S.I.D. for others five months (T2). FP was given daily between 7:00- 9:00 AM and 4:00-6:00 PM during the first six months (T1) and once daily (AM) for the remaining five months of the study (T2).

### Clinical respiratory score (CRS)

A clinical respiratory score previously reported [57, 61] was used. This score consider two components: Nasal flaring and abdominal movement recorded on a scale from 1 to 4. For nasal flaring, the score ranged from 1(no flaring) to 4 (continuous flaring during each respiration), while for the abdominal component the range is 1 (no abdominal movement during

breathing) to 4 (marked abdominal movement during breathing. The sum of the score of both components may totalized a total clinical respiratory score of 2 (no signs) to 7 or 8 (severe clinical respiratory signs). A strong correlation has been established between severity pulmonary disease, clinical respiratory score and pulmonary function [57, 61]. This score was recorded by the same veterinarian all way through the study in blind fashion without any knowledge about lung function respiratory measurements. This clinical respiratory score has been proved to have a good correlation with some of the lung function measurements, such as dynamic elastance, however some minimal obstruction degree may arise before the clinical signs are more evident [57].

**Cortisol analysis**

Blood samples for cortisol were always collected between 8:00-9:00 AM on d 12 and 10 before treatment in order to establish baseline cortisol values. Blood samples were also collected between 8:00-9:00 AM on d 7, 28, 80,160, 200, 250, 290 and 320 under treatment regimen before FP was administrated. Blood was drawn from the jugular vein using a 18-gauge x 1 1/2 inch vacutainer needle in a red cap vacutainer® tube without anticoagulant. After 60 min at room temperature (15°C) blood samples were centrifuged (SL16R, Thermo Scientific, Canada) at 1000 g, 15°C for 20min (1900 RPM). Serum samples were separated, aliquoted, and stored a -80°C until analysis. Serum cortisol was determined by chemiluminescence enzymatic immunoassay technique (CEIA, Immulite 1000® Siemens, Canada), according to manufacturer protocol [3]. Blood sampling processing was always done keeping same timing (during 2 hours after collection), minimal stress to have consistency, avoiding circadian variability and stress-related variation [8].

**Statistical analysis**

The method applied for cortisol levels was a repeated measures analysis based on the analysis of variance (ANOVA lineal model), and data were fitted to a model that included the effects of treatment (RAO-FP and RAO-Control), effect of phase (un-equally spaced time (seasonal effect)), and treatment × phase interaction. The model for the design is as follows:

$$Yijk = \mu + \sim j + \quad k + (\sim\!: )jk + Eijkl$$

i = 1, 2, 3…11 (horses) j = 1, 2 (treatment)

k = 1, 2, 3…9 (Phase of experiment)

where Yijk = the variable of interest for the horse i, assigned to treatment j, and phase k; μ = the overall mean; ~j = the treatment effect; k = the phase effect; (~: ) jk = the treatment × phase interaction; and Eijk = the error term. To use this model, we must assume that Eijk, is the random error associated with the i horse in the j treatment at k phase. Data were corrected by sequential Bonferroni's test to correct comparison-wise ~ levels, differences were determined significant at P<0.001.The linear model for the cortisol repeated measures over time (9 levels) as within-subject factor and group (two levels) as between subjects factor, indicated a significant effect of group all time combined (P= 0.03) and all time groups combined (P <0.0001). A priori contrasts with the sequential Bonferroni correction showed no difference between the average of the two groups at 0, 2, 7, 200, 250 and 290. At time 28, 80 and 160, the average was significantly lower in the fluticasone group than in the control group. A ANOVA lineal model analysis for CRS (Clinical respiratory score) was also done over time (3 levels) as well as within-subject factor and group (two levels) and means values comparisons among each time points, groups and treatment were done using t-test. Graphics were done using Sigmaplot®.

## RESULTS AND DISCUSION

**Clinical respiratory score (CRS)**

In this study CRS correlated very well with RAO symptoms. CRS importantly improved at the end of the study in both groups whether they were or not on FP treatment. At the beginning of this study (T0), when all horses were stabled in barn, fed with hay and kept in a hay-dusty environment, all of them showed RAO symptoms. However, in the second part (T1), after FP or environment control (Hay or pelleted feed) were established, CRS slightly decreased only in RAO-control. On pasture (T2) CRS median values in both groups of horses were importantly lower and becoming even clinically normal CRS in both groups. Similarly, CRS from T0 toT2 significantly decreases in both groups (P<0.001) (FIG.1).

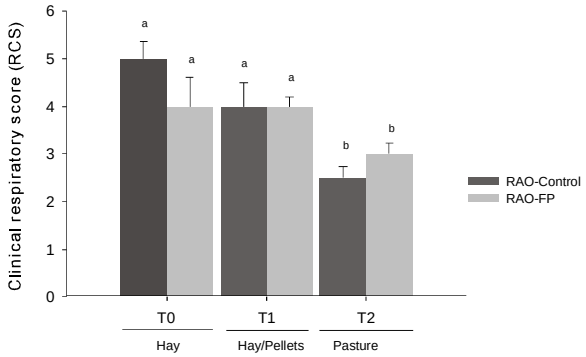


**FIGURE 1. CLINICAL RESPIRATORY SCORE (CRS) MEDIAN VALUES IN RAO-CONTROL AND RAO-FP (FLUTICASONE PROPIONATE).**

**Cortisol**

Cortisol levels at T0, before the FP treatment initiation, were similar between groups; (FIG. 2). Once FP 2000 µg/horse B.I.D started (T1) and environmental management were implemented (hay or pellets), cortisol values on d 7 were also similar between groups. However, cortisol levels were significantly lower in RAO-FP horses compared with RAO-control horses on d 28 (P<0.001); 80 (P<0.001) and 160 (P=0.002) (FIG. 2) and even versus baseline values (FIG. 3).

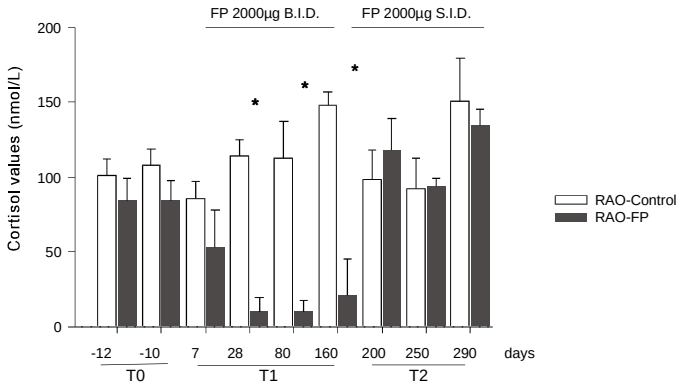


**FIGURE 2. RAO-CONTROL AND RAO-FP CORTISOL VALUES (MEDIAN +/- STD. ERROR) DURING THE ELEVEN MONTHS OF THE STUDY.**

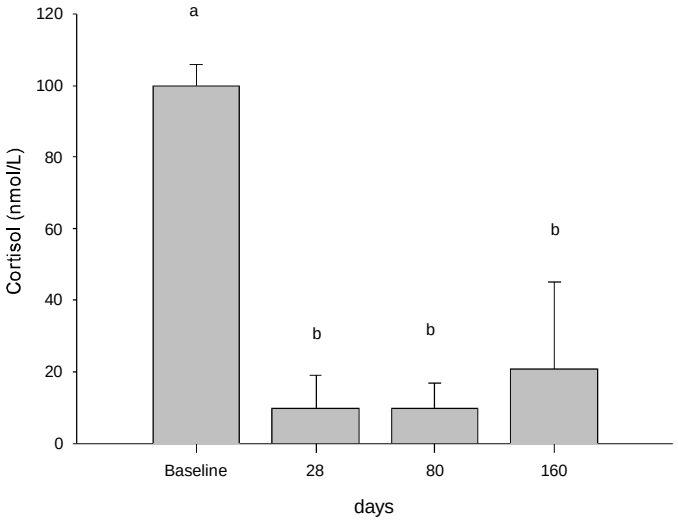


**FIGURE 3. COMPARISON BETWEEN BASELINE CORTISOL VALUES (T0) (MEDIAN VALUES +/- STD. ERROR) VS. CORTISOL VALUES OF RAO-FP HORSES (2000µ/HORSE/TWICE-A-DAY) (T2) ON DAYS 28, 80 AND 160 DAYS.RAO-CONTROL AND RAO-FP CORTISOL VALUES WERE POOLED AND TAKEN AS A CORTISOL BASELINE VALUE, BEFORE FP STARTED (T0).**

During six months, all horses were kept always indoor, and then both groups were moved to pasture for the remaining time (5 months) of the study (T2). Cortisol levels on d 200 and 250 in RAO-FP group under a 2000 µg FP /horse/S.I.D. declined and were slightly higher than baseline values; howeverno differences were found between groups. Similarly, cortisol on d 290 (at the end of the study) was slightly higher than baseline values in both groups. However, not differences were found between groups, maintaining the same trend until the end of the study (FIG. 2).

**Cortisol in RAO-FP group**

Cortisol levels on d 160 and. 200 increased (rebound effect) during this period, matching with the FP dose adjustment from 2000 µg/horses B.I.D. to 2000 µg/horse S.I.D. (FIG.2). Cortisol in RAO-FP horses during this period start to follow the same seasonal variability as seen on RAO-control horses (FIGS. 2, 3, 4a and 4b).

Cortisol level on d 250 vs. 290 followed a similar seasonal pattern in both groups, however in RAO-FP group with some delayed distortion. Seasonal cortisol change on RAO-FP horses is in a lesser degree compare to RAO-control horses (FIGS. 2, 3, 4a and 4b). This let suggest that FP might have a greater dysregulation impact on HPA and more evident suppression effects under a FP 2000 µg/horse B.I.D. regime than 2000 µg/horse S.I.D. (FIG. 5 and 6).

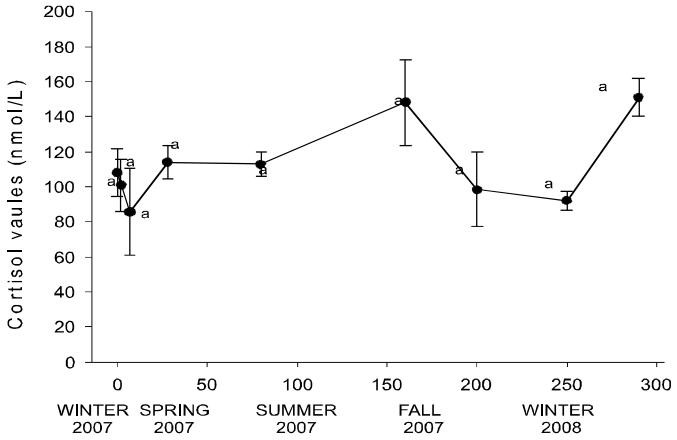


**FIGURE 4A. CORTISOL CIRCANNUAL VALUES (MEDIAN +/- STD. ERROR) IN RAO-CONTROL HORSES ALONG 290 DAYS.**

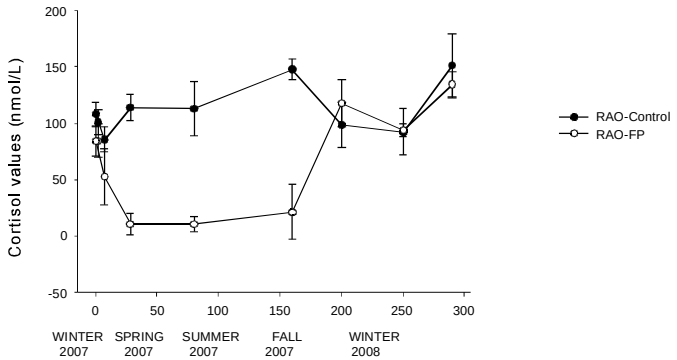


**FIGURE 4B. CORTISOL CIRCANNUAL VALUES (MEDIAN +/- STD. ERROR) IN RAO-CONTROL HORSES AND RAO-FP HORSES ALONG 290 DAYS.**

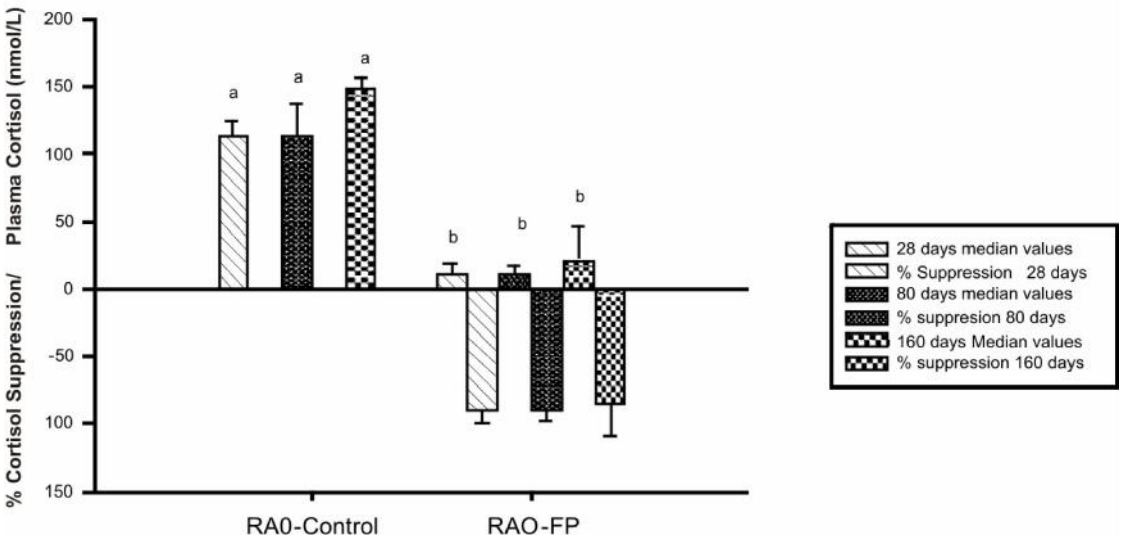


**FIGURE 5. CORTISOL VALUES AND CORTISOL SUPPRESSION (MEDIAN VALUES +/- STD. ERROR) BETWEEN RAO-CONTROL AND RAO-FP (2000µg/HORSE B.I.D.) HORSES.**

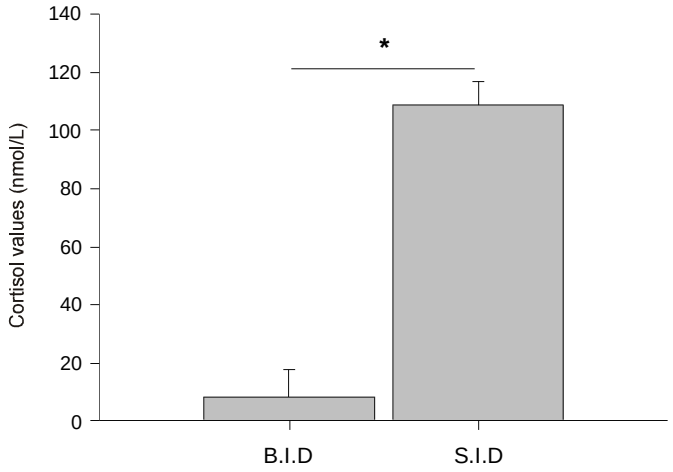


**FIGURE 6. SERUM CORTISOL LEVELS (MEDIAN VALUES +/- STD. ERROR) BETWEEN RAO-FP HORSES TREATED WITH 2000µg B.I.D. VS. S.I.D. FOR EACH REGIMEN PERIOD THREE (3) SAMPLING TIMES WERE POLLED BY**

Cortisol suppression in RAO-FP group on d 28, 80 and 160 ranged between 85.67 ± 24.30% and 90.96 ± 9.34% (median values ± std. error), compared to cortisol values in RAO-control during same period of time (FIG. 5). Nonetheless, cortisol suppression was reversible. HPA axis was still able to re-establishing a cortisol plasmatic secretion in the second half of the therapeutic regime, and caused a "rebound effect" in cortisol levels with a FP 2000 µg/horse/S.I.D. therapeutic regimen (FIG.4b and 6).

Several methods (e.g. radioimmunoassay, immunoaffinity, chromatography, gas chromatography, immunoassay, HPLC, LC-MS and (LC/TSQ-MS/MS) have been used to measure steroids levels post-race in urinary and plasma samples in horses, either for forensics and confirmatory purposes. CEIA technique sensitivity is comparable to radioimmunoassay having the advantages that do not require special facilities for radioactivity handling [67]. Moreover cortisol assessment is one of the most practical endocrinology test in horses [14, 18, 21, 35, 55, 61, 62]. Nevertheless, dexamethasone suppression test (DST) is the gold standard test in horses, for diagnosing HPA dysregulation due to chronic elevation of circulating endogenous glucocorticoids, in horse suffering of PPID (pituitary pars intermedia dysfunction) [19, 20, 65 ].

In any case, blood sampling in horses for cortisol determination should be done with the same timing, minimal stress, good consistency and using same methodology, repeatability and standardization, in order to avoid circadian variability [24] as well as, stress-related variation [8], in order to have a better picture of the real plasma cortisol levels.

Several methods for assessment of the secondary effects of inhaled steroids treatments in a equisystemic basis have been proposed, thought the screening of serum morning cortisol levels and hourly serum cortisol measuring [40, 41]. In humans, also AUC (area under curve) of 24-hrs cortisol urinary profile for estimating cortisol suppression has been used as a method for inhaled glucocorticoids systemic availability assessment as well as the potential for causing systemic side effects [41].

Cortisol values ranged between 30–395 nmol/L in healthy adult horses at rest [1, 18], in agreement with most domestic animals [18]. Similarly, cortisol in healthy horses under stress by shipping or transport [8] ranged between 103-278 nmol/L, without differences related to sex or age, however values were higher in May and June vs. October.

Interestingly, cortisol is expected to be higher during stressful situations, like the RAO acute phase (266.66 nmol/L±86.66), only decreasing after RAO symptoms improve-

ment or FP cortisol suppression action or both [55], however cortisol values in this study were not related with CRS improvement because cortisol values in RAO-control increase in clashing to the weakening seen in other short FP studies. Cortisol in RAO-FP horses significantly decreases and seems to be more dependent on FP suppression action than on symptoms improvement.

Regardless, this trend seen in others short studies with RAO horses treated with FP, in this study RAO-horses (pooled cortisol baseline values) were (100.50 nmol/L ± 6.00), somewhere following a seasonal variability having the higher cortisol median values in September (Fall) (148 nmol/L ± 8.73) and January (late winter) (151 nmol/L ± 28.14) (FIG.4b). However, not differences between these two periods were found as in other study with healthy horses during the same periods [18].

**Seasonal Variations**

This clinical study started in late winter (2007), when d are getting longer and that could explain an important increased in plasma cortisol in RAO-control on d 7 (April) vs. d 28 ($P<0.03$) (FIG. 4a). Then, there was an increase in early summer followed with a decrease on d 200 (late fall, October) and on d 250 (early winter, December). At the end of the study, there was an increase again on d 290 (late winter January 2008), probably preparing the body by handling better the few available energy sources for next food scarcity season. All this seasonal variation in daylight length has a great impact in the cortisol secretion (circadian and circannual rhythms) (FIG. 4a).

RAO-control horses have normal seasonal fluctuation pattern on circannual cortisol values along the one-year study. The highest cortisol values were recorded in early fall and late winter (FIG. 4a). Comparing these normal circannual fluctuations on cortisol values of RAO-control horses to the cortisol values of RAO-FP horse indicate that seasonal cortisol levels behaved in the same way in both groups and the only reason of disruption on seasonal cortisol levelsin RAO-FP groupis justdue to FP treatment. The only disruption seen on normal circannual variation (established by comparison to cortisol levels on RAO-control) is on days 28, 80 and 160 by FP 2000 µg/horse/B.D.I. on RAO-FP group Therefore, the possibility exists that any variations on circannual and probably on circadian cortisol values in RAO-FP horses are not environmental or RAO-related instead may be attributable to the FP treatment. In summary, FP 2000 µg/B.I.D. is capable to disrupt the synchronism in the HPA axis that is reflected in the alteration of normal circannual and probably even circadian fluctuations in those horses suffering of RAO and treated with FP (FIG. 4b). Despite cortisol measurements were done only once a day (morning assessment), they always remained lower in RAO-FP both on the daily and seasonal analysis, probably because both circadian and circannual, are affected on d 28, 80 and 160 d due to FP in treated horses.

FP is only capable to induce important cortisol suppression through a regimen of 2000 mg/horse B.I. D. ($P<0.001$). Since FP half-life is about 13-14 (h), FP 2000 µg/horse S.I.D. is unable to cause and maintain HPA suppression (FIG. 5 and 6).

The findings suggest that more cautious approach may be consider with long term FP usage to manage and prevent symptoms in RAO-aged horses, as well as how relevant are FP dose, time and treatment interval on the appearance of any side effects on the HPA axis [31]. Therefore, cautious should be taken with the FP 2000 µg B.I.D. approach for a long term due to cortisol suppression consequences. Furthermore, FP 2000 µg S.I.D. in RAO management is clinically impractical to control symptoms. Environmental control by itself would be a better and a more advantageous strategy than FP long-term treatment in preventing and controlling RAO symptoms, without increasing the likelihood to cause HPA suppression. A special consideration in using FP may be taken into account cortisol variability along seasons.

In haled steroids are believed to cause less systemic harm, however, they have been associated with adrenal suppression too [35, 58] For instance, bectomethasone dipropionate (Beclo) is capable to produce neutropenia in the BAL of treated horse [59].

Several drugs and medicaments (e.g. etomidate, ketaconazole and rifampin) are involved in cortisol suppression through 11- hydroxylase enzyme inhibition, interfering with last step in active cortisol synthesis, and causing adrenocortical insufficiency [17, 22], that might explain some of the actions observed in RAO aged horses treated with FP.

Steroids anti-inflammatory actions and secondary effects pathways on cellular modulation share the same receptors, nonetheless receptor proportions for any those actions appears be different among tissues making some tissues more prone to therapeutics than deleterious actions or vice versa [5-7, 23, 31].

Since RAO is an inflammatory pathology, then there is the general assumption that its inflammation nature explains most of the changes on pulmonary mechanical properties. Therefore, it is also assumed that ameliorating and controlling inflammation may have a beneficial effect on pulmonary mechanical properties as well as in clinical symptoms. However, from an evidence–based on veterinary medicine (EBVM) point of view those assumptions are not totally convincing [74]. For example, in most studies dexamethasone (Dexa) efficacy in ameliorating pulmonary resistance, using a variety of doses and routes of administration seems to be more related to the timing of the measurement after treatments and the statistical power of the study [49, 56].

Similarly, neither the efficacies of inhaled steroids (e.g. Beclomethasone and Fluticasone) nor their anti-inflammatory properties on various aspects of RAO reported in several peer-reviewed publications, have been particularly convincing from an EBVM point of view [74]. For instance, beclometha-

sone dipropionate (Beclo) (500 mg twice daily) effectively cause a fast improvement in respiratory score in a short time as 10 d that do not translate in an evident effect in pulmonary resistance. The Beclo treatment 1320 mg (high dose) twice daily caused a fast improvement in clinical score and displayed correlated changes in pulmonary resistance only after 10 days of treatment [61, 74].

For instances FP in a double blind, randomized placebo-control with a dose of 1980 µg, given twice-a-day, delivered with a meter-dose inhaler and Aeromask®, showed more strong evidence from EBVM. Nevertheless FP action was not complemented with evident decrease in airway inflammation [14].On the other hand, a crossover study showed that FP is more effective for RAO symptoms prevention than treatment. FP 6000 µg twice-a-day may have a beneficial for initial treatment of RAO and used in prevention as Dexa in RAO-affected horse in remission, however this high-dose is less consistently than Dexa for the short term treatment [55].

Among all the studies using inhaled FP, only two studies reported cortisol suppression: one controlled crossover study in RAO-horses [55] and another in RAO-free horses. The first one [55], compared 3000 µg and 6000 µg of FP B.I.D. during 3 and 7 d, respectively and showed that both FP treatments are capable to significantly suppresses cortisol as shortly as 72 hours (3000 µg B.I.D.) and even after 8 d of initiating the FP treatment (6000 µg B.I.D.), respectively. The second one [35], reported adrenal suppression (on d 1, 3 and 5) in RAO-free symptoms horses after a treatment with FP using a persistent dose 1500 µg (medium dose) by horse/twice-a-day for 7 d. However, in that study the adrenal suppression caused by FP was not statistically important, probably due to the lower FP dose, small number of animal, the heavy body weight of horses (Dutch warmblood) used in that study or combination all these factors [35] (TABLE I).

Other study in RAO horses [35] used a starting high dose of FP 1980 µg/ horses/ twice-a-day for 7 d, followed for another 14 d period, with a decreasing dosage and frequency approach, from twice-a-day to one-a-day for 7 d and another week with alternated administration did not showed cortisol suppression or HPA dysregulation (TABLE I).

Beclo in RAO horses even a 528 µg (low dose) B.I.D for 6 d and 1320 µg B.I.D for 7 d, are both capable to cause adrenocortical suppression in RAO-horses under both treatments approach regimen [61] (TABLE I).

RAO-horses seem to be more susceptible to cortisol suppression actions of Beclo, on a dose equivalent basis, compared to the same dose used in asthmatic patients. Beclo dose threshold in horses capable to induce suppression is as low as 528 µg B.I.D. Nevertheless a 500 µg Beclo is enough to achieve comparable improvement in pulmonary function, as 1000 and 1500 micrograms Beclo does with fewer suppression of endogenous cortisol. Controversially, another study found that 500 µg of beclo B.I.D. is capable to improve clinics sings and pulmonary function [12, 13, 61].

Dexa single dose in horses causes a long-lasting effect in endogenous cortisol (96h) as well as important decrease in lactate (36h) and glucose (60h) after intravenous administration [68].

FP systemic bioavailability predominantly depends on its rate of absorption from the lungs. FP contrary to other inhaled drugs does not require tissue conversion as Budesonida does. FP is twice potent than budesonide and Beclo [4], FP also has 20-times superior GC receptor affinity than Dexa [4, 23].

*TABLE I*
**CONTROLLED STUDIES IN HORSES USING INHALED STEROIDS**

Controlled studies in horses using inhaled steroids

| Study | Inhaled steroid and dose | Horse | Cortisol suppression or HPA axis dysregulation |
|---|---|---|---|
| Laan et al. 2004 [62] | Fluticasona propionate<br>1500µg B.I.D. for 7 days | RAO-free horses | Yes |
| Couetil et al. 2005 [14] | Fluticasona propionate<br>-1980µg B.I.D. for 14 days<br>-1100µg S.I.D. for 7 days<br>-1100µg every two Days for 7 days | RAO horses | No |
| Robinson et al. 2009 [55] | Crossover study<br>-FP 3000µg and 6000µg B.I.D during 3 days<br>-FP 6000µg B.I.D for 7 days | RAO-horses | Yes |
| Rush et al. 2000[61] | Beclomethasone<br>1320µg B.I.D. for 7 days | RAO-horses | Yes |
| Rush et al. 2000 [61] | Beclomethasone<br>528µ B.I.D. for 6days | RAO-horses | Yes |

Moreover, FP causes two time greater adrenal suppression than budesonide on a microgram equivalent basis in asthma patients [23].

FP oral bioavailability is as lower as less than 1%. Most oral FP is absorbed (90%) through oral tract, however higher receptor affinity and solubility fluctuates according to the propellant used on the meter-dose container [23].New propellant HFA could potentially improve pulmonary deposition of Beclo, with fewer side effects due to oral deposition and subsequent absorption [72].

In human, fluticasone absorption is greater in normal patients without pulmonary obstruction than in asthmatic patients [4]. Similarly, short-term studies reveal that the fluticasone absorption is greater in healthy horses than in RAO affected horses, using cortisol suppression as a parameter [14, 35], which could indicate a greater drug absorption surface area in healthy animals. Although, inhaled corticosteroids systemic effects may also arise from G.I. absorption since a large dose proportion using a metered dose inhaler is deposed in oropharynx [58].

Systemic steroids in horses have been associated with muscular emaciation, weakness, hyperglycemia, hypokalemia, polyuria, polydipsia, immuno-suppression and lameness [26]. Inhaled steroids are believed to cause less systemic harm, however, they have been associated with adrenal suppression too [35, 62, 63].

Others therapeutics options designed to cause less collateral effects have been tested, like: nitro-steroids, NO-Prednisolone (NCX1015) or NO-hydrocortisone (NCX-1004), aliphatic or aromatic molecules derived from nitric oxide [46, 48], selective glucocorticoids receptors agonists (SEGRAs) [46, 48]. Unfortunately those preliminary promissory in vitro results have not been translated in good clinical results.

Some *in vitro* studies in horses, have found not immunology effects fluticasone-related on lymphocytes function after a long treatment with fluticasone [16]. FP in dogs (*Canis familiaris*) doesn't have overall impact on immune system regardless cortisol suppression [11]. Nevertheless HPA activation in rats due to stress causes an increase in endogenous cortisol, immunosuppression and dysregulation on Th1/Th2 cytokines profile [73]. RAO by itself is a chronic stress condition and exogenous FP long-term exposure over HPA axis, are not entirely known. Moreover, a pituitary pars intermide distalis (PPID) horses have ~–MSH adaptive mechanism to cope with pro-inflammatory status [42].

Geriatric horses are suspected of having two particular inflammatory conditions, RAO and pituitary pars intermide distalis (PPID) in a concomitant way, making them more prone to have a more pro- inflammatory profile or to shift their general cytokine and hormone profile in an extremely unpredictable way after a prolonged inhaled corticosteroid therapy to counterattack this pro-inflammatory status [35, 42].

Anecdotally in this study, evening cortisol values after dexamethasone suppression test (DST) and pathological findings of pituitary adenoma (macroadenoma) [43] were diagnosed in two horses, one belonging to each group of horses on the study. The RAO-control cortisol values were still higher after DST (data not showed). However, as expected in a typical horse with (PPID) the RAO-control horse was always unresponsive to Dexa action, and its cortisol values were higher than RAO-FP that was RAO-free symptoms at this point. Controversially, cortisol levels from this last are far below detection levels after DST compare to RAO-control. These results provide some hints to speculate that the FP cortisol suppression is strong enough and FP is still capable to suppress pars distalis and even supplementary pars intermedia steroidogenesis in RAO-FP group regardless the adenomatous pituitary. For instances, secondary hypoadrenocorticism associated PPID treatment with pergolide has also been reported [45], that lets us rethink the potential FP impact on HPA axis.

## CONCLUSION

FP 2000 µg B.I.D causes suppression in serum cortisol on days 28, 80 and 160, which seems to be reversible when dosage was decreased by half. Unfortunately, a comprehensive endocrine assessment was far beyond the scope of this study. This clinical study was not able to rule out whether the HPA axis dysfunction by FP on cortisol suppression was caused from adrenal or central source. This clinical study was not intended to investigate cortisol circadian variations; it was designed for the study of cortisol circannual variation in RAO horses FP-related.

Whether FP S.I.D. is effective in control RAO symptoms remains unclear due confounding factors: FP and environmental management. Hay dust pollution was improved in the second-half part of this study since horses had less exposure to dust or spent more time outdoors. RAO symptoms in both groups might have a self-resolving nature. Morning cortisol level is not different among groups; however, evening cortisol after DST was still lower in RAO-FP (data not published) that means that this dose is still able to suppress the HPA axis. Negative feedback loop dependent upon steroid dose, potency and timing.

Perhaps, FP may be more advantageous due its local actions. Nonetheless, compare to humans, horses might be more susceptible on a body weigh basis, since FP and Beclo human dose cause suppression in adult horses. FP potential causing iatrogenic insufficiency should always be considered as an outcome of steroid supplementation, moreover adrenal exhaustion syndrome and cortisol insufficiency has been described in racehorsesand critical foals associated with inflammatory dysregulation.

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