Horses fed meal contaminated with mycotoxins performing short-term exercises[1]

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ABSTRACT.- Braga A.C., Almeida F.Q., Silva V.P., Almeida M.I.V., Keller K.M., Diegues V.A., Thomes F.R. & Rosa C.A.R. 2020. **Horses fed meal contaminated with mycotoxins performing short-term exercises**. *Pesquisa Veterinária Brasileira 40(9):717-725*. Programa de Pós-Graduação em Ciência, Tecnologia e Inovação em Agropecuária, Universidade Federal Rural do Rio de Janeiro, Rodovia BR-465 Km 7, Seropédica, RJ 23890-000, Brazil. E-mail: auro_vet@hotmail.com

This research was carried out to evaluate the effect of mycotoxins on the performance of horses through physiological parameters, and hematology and serum biochemistry analyses. The essay lasted 40 days, with 12 days for adaptation and 28 days of experimentation. In the experimental stage, the horses were distributed in a completely randomized design, with three treatments with four animals each. The treatments used were 0 (control), 50 ppb and 100 ppb of Aflatoxin B_1 (AFB_1) added to a concentrate in a basal diet. The basal diet contained mycotoxins from feedstuffs naturally contaminated. The exercise test was performed over the 21th day of the experimental stage. The exercise consisted in an interval training test with a warm-up of 17 mins at a trot followed by three gallops of 450m/min. The heart rate was monitored between the gallops. Before the exercise test and immediately after the third gallop, the physiological and blood parameters were evaluated, and continued up to 48 hours after the exercise. The results of the physiological, hematological and biochemical parameters were submitted to analysis of variance (ANOVA) and compared by the Tukey test at 5% of significance. The presence of AFB_1 in the diet influenced the alkaline phosphatase activity, which presented higher values in horses fed diet with inclusion of 100 ppb AFB_1, suggesting a hepatotoxic activity associated with the others mycotoxins naturally present in the feedstuffs.

INDEX TERMS: Horses, contaminated meal, short-term exercises, alkaline phosphatase, exercise, mycotoxins, morphology.

RESUMO.- [**Efeitos das micotoxinas na performance de equinos submetidos a exercícios de curta duração.**] Esta pesquisa foi conduzida para avaliar o efeito de micotoxinas no desempenho de equinos com avaliações fisiológicas e análises hematológicas e da bioquímica sérica. O ensaio durou 40 dias, com 12 dias de adaptação e 28 dias de experimentação. Na fase experimental, os equinos foram distribuídos em delineamento inteiramente casualizado em três tratamentos,

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com quatro animais cada. Os tratamentos utilizados foram 0 (controle), 50 ppb e 100 ppb de Aflatoxina B_1 (AFB_1) adicionada ao concentrado de uma dieta basal. A dieta basal continha alimentos naturalmente contaminados por micotoxinas. O teste de desempenho foi executado no 21° dia da fase experimental por meio de teste intervalado consistindo em aquecimento ao trote por 17 minutos, seguido de três galopes de 450m/min. A frequência cardíaca (FC) foi monitorada entre os galopes. Antes do exercício e imediatamente após o terceiro galope, os parâmetros fisiológicos e sanguíneos foram avaliados e continuaram sendo monitorados até 48 horas após o exercício. Os resultados dos parâmetros fisiológicos, hematológicos e bioquímicos foram submetidos à análise de variância (ANOVA) e comparados pelo teste de Tukey a 5% de significância. A presença de AFB_1 na dieta influenciou a atividade da fosfatase alcalina, que apresentou valores mais elevadas na dieta com inclusão de 100 ppb de AFB_1, sugerindo uma atividade hepatotóxica associada às outras micotoxinas naturalmente presentes nos alimentos.

TERMOS DE INDEXAÇÃO: Micotoxinas, equinos, exercícios de curta duração, fosfatase alcalina, micotoxinas, morfologia.

INTRODUCTION

Several mycotoxins have been identified in foods and diets for humans and animals in Brazil. The importance of aflatoxins (AF), fumonisins (FB), ochratoxins (OTA), zearalenone (ZEA) and trichothecenes should be emphasized due to their occurrence and also their high toxigenic potential for farm animals (Baldissera et al. 1993, Franco et al. 2019). In addition, the additive or synergistic effect between different toxins, which increase or alter their effects on animals, should be considered. Although mycotoxins have several adverse effects on animal health, little is known about their impacts in the horse nutrition (Liesener et al. 2010, Schumann et al. 2016). Raymond et al. (2005) pointed out that, especially for sport horses, chronic intake of low mycotoxin levels in diets can affect performance without animals showing typical clinical signs of mycotoxicosis.

Aflatoxins are a class of carcinogenic mycotoxins produced by *Aspergillus* fungi and are widely distributed in nature, especially in a variety of food commodities. Aflatoxin B_1 (AFB_1) is confirmed to be the most toxic of all the aflatoxins and has been well-characterized to lead to the development of hepatic cellular carcinoma (HCC) in humans and animals. The occurrence of these mycotoxins, alone and in combination, represents a real risk for human and animal health (Bertero et al. 2018, Rushing & Selim 2019).

Although the importance of mycotoxins in public and animal health is recognized, there is no uniform regulation of mycotoxin limits in foods in the different countries, particularly in Mercosur countries (Brazil/Argentina), (Mercosul/GMC 2002). The Brazilian legislation (Brasil 1988) establishes the maximum limit of 50µg/kg for aflatoxins in any feedstuffs used directly or as an ingredient in diets for animals but does not specify limits for other mycotoxins. This research was carried out to evaluate the inclusion of aflatoxin B1 (AFB_1) in the diet and to verify the effects on metabolism and performance of horses.

MATERIALS AND METHODS

The experiment was conducted at the Army Sergeant School (ESA), latitude 21°42'15.6'' South and longitude 45°15'18.9'' West, of the Brazilian Army, in Três Corações, Minas Gerais State. The assay was performed in accordance with the principles of ethics in experimentation approved by the committee on ethics in animal experimentation from the University: CEUA-UFRRJ Number 1212031017.

Experimental period. The total experimental period was 40 days, divided into two stages: one was the pre-experimental or adaption stage of 12 days and the other was the experimental stage of 28 days. A total of 12 adult Brazilian Sport Horses (9 females and 3 males), aged between 5 and 8 years old with an average body weight of 458.02±22.09kg were used. The research was conducted in a completely randomized design, with three treatments with four animals each, and the animal was the experimental unit. The experimental treatments used were: Treatment I = control diet (0µg/kg of AFB_1); Treatment II = diet with 50µg/kg of AFB_1; and Treatment III = diet with 100µg/kg AFB_1. The AFB_1 was added to the basal diet (Table 1). The experimental basal diet was composed by Tifton hay (*Cynodon* spp.) and alfafa (*Medicago sativa*), commercial concentrate (S Line -280, Royal Horse®), mineral salt and water *ad libitum*, elaborated according to the NRC (2007) for mature horses. The horses remained free in their paddocks during the day and were housed in individual stalls at night. The animals were fed according to the schedule of the Military Unit, which is 2kg of concentrate meal at 05:00 a.m. and 2kg at 17:00 p.m., alfafa hay at 10:00 a.m., and tifton hay at 13:00 p.m. and 20:00 p.m. Every day during the experimental period at the 17:00 p.m. feeding time, 1kg of concentrate feed with AFB_1 was prepared and given to the animals, according to the treatment. Before the adaptation period, all feedstuffs were analyzed, and the concentrations of mycotoxins in the basal diet were: 5.620µg/kg of DON, 708µg/kg of ZEA and 560µg/kg of FB_1.

Clinical conditions. Before, during and after the experimental period, clinical, hematological and biochemical analyzes of the horses were carried out to check the health conditions, especially regarding the presence of liver damage. Throughout the experimental period, the clinical conditions of the animals were monitored, daily, by a veterinarian.

Aflatoxin production and estimation. The aflatoxin B_1 was produced by the Mycology Laboratory of Federal University Rural of Rio de Janeiro from fermenting rice with a spore suspension of *Aspergillus parasiticus* (NRRL 2999), according to Shotwell et al. (1966). The AFB_1 content was extracted and purified through a Mycosep® Aflazon column (Romer Labs Diagnostic GmbH., Tulln,

Table 1. The average composition (g or µg /kg on dry matter basis) of the experimental diets with different inclusions of AFB_1

Diet Components	Treatments		
	T I (Control)	T II (50 ppb AFB_1)	T III (100 ppb AFB_1)
Tifton hay/Alfalfa (g)	7.400	6.900	6.600
Commercial concentrate (g)	4.000	4.000	4.000
Mycotoxin concentration (µg)			
Aflatoxin B_1 (AFB_1)	0	50	100
Zearalenone (ZEA)	2.758,1	2.687,5	2.655,2
Deoxynivalenol (DON)	21.564,7	20.689,9	20.289
Fumonisins B_1 (FB_1)	2.240	2.240	2.240
Total (g)	11.400	10.900	10.600

Austria) as per the manufacturer's instructions and an aliquot (200μl) was derivatized with 700 μl trifluoroacetic acid-acetic acid-water (20:10:70, v/v). The derivatized aflatoxin was quantified by High Performance Liquid Chromatography (HPLC), at the "Laboratório de Micologia e Micotoxinas" of "Universidade Federal de Minas Gerais", following the methodology proposed by Trucksess et al. (1994).

Aflatoxins were quantified by injecting 20μL of extract from each vial into an HPLC system consisting of a JASCO model LC-2000 pump (Tokyo, Japan) connected to a JASCO model FP-2020 programmable fluorescence detector and a data module JASCO model 6937-J041A (ChromNAV). Chromatographic separations were performed on a stainless steel, C18 reversed-phase column (Supelcosil™ LC-ABZ, 150 x 4.6mm, 5μm particle size) connected to a precolumn (Supelguard™ LC-ABZ, 20 x 4.6mm, 5μm particle size). The mobile phase was water:methanol:acetonitrile (4:1:1, v/v/v) at a flow rate of 1.0ml min^{-1}. Fluorescence of aflatoxin derivatives was recorded at excitation and emission wavelengths of λ 360nm and λ 460nm, respectively. Standard curves were constructed with different levels of AFB_1. This toxin was quantified by correlating peak heights of sample extracts with those of standard curves. The detection limit of the analytical method was 0.4ng g^{-1}.

The concentration of AFB_1 yielded was 4.25mg/kg and it was diluted daily in the concentrate feed to obtain the desired concentrations according to the equation: Ci xVi = Cf x Vf, (where Ci and Cf are the initial and final concentrations and Vi and Vf, the initial and final volumes).

Performance evaluation. The performance was assessed through an exercise test at the 21th day of the experimental stage. The average temperature during the day was 21.5°C, with a minimum of 12.2°C and maximum of 28.1°C and the relative humidity was 62.4%. The animals performed standard exercise training for 50 minutes, 5 days per week, during three weeks of the experimental stage. The training program of walking and trotting had been intercalated with flexion movements. For the exercise test, the animals were randomly split into 3 groups with 4 animals each. The test started at 9:00 a.m. and finished at 12:06 a.m. on the same day; they were performed sequentially for each group. The test consisted of a 17 min warm-up of walking and trotting followed by three sets of gallops at a speed of 450m/min for three minutes each. In the intervals between the gallops, the horses were monitored for heart rate (HR), and when the heart rate returned to 60-70bpm the animals moved on to the next gallop. In order to evaluate the performance of the animals in the test, the heart rate, the respiratory frequency was recorded. Blood samples were taken to analyze the globular volume, glucose, lactate and blood biochemistry. These variables were obtained before the exercise test, and after the third gallop and continued up to 48 hours after the test as described: M0 (Pre-exercise), M1 (Immediately after the exercise test), M2 (10min), M3 (20min), M4 (60min), M5 (3h), M6 (6h), M7 (12h), M8 (24h) and M9 (48h).

Heart rate and respiratory rate were recorded with a stethoscope (Mod LittmannMR). Blood samples were collected using a vacuum collection needle (BD®) and vacuum tubes with EDTA (BD Vacutainer®) for the complete blood count (CBC). Vacuum tubes with sodium fluoride (BD Vacutainer®) were used to determine glucose and lactate measures and vacuum tubes without anticoagulant (BD Vacutainer®) were used to obtain blood serum samples, for biochemical determination of enzymatic activity, total proteins and fractions, cholesterol, urea and creatinine measures. After collection, the samples were centrifuged at 7.000rpm for 10 minutes to separate the plasma and serum. Aliquots of both plasma and serum of 1.0mL were pipetted automatically and stored at -20°C in 1.5mL polypropylene tubes until

required for analysis. Subsequently, the analyzes were performed by the enzymatic/colorimetric method in a spectrophotometer SB 190 (CELM® Cia Equipadora de Laboratórios Modernos - CELM. Brazil). Labtest® kits were used for all analyzes.

Statistical analysis. The results were evaluated for normality by Lilliefors test and the homogeneity by Cochran and Bartlett test. Data of the hematological, biochemical and physiological variables were submitted to analysis of variance (ANOVA) in a split-plot scheme with the levels of inclusions of AFB_1 in the diets evaluated in the parcels and the exercise test times evaluated in sub-parcels. The means were compared by the Tukey test, at 0.05 significance level, using the Statistical and Genetic Analysis System (SAEG 2007).

RESULTS

Occurrence of mycotoxins in feedstuffs

The mycotoxins observed in the feedstuffs of basal diet were: DON, 5.620μg/kg, in Tifton and alfalfa hay and commercial concentrate; ZEA, 708μg/kg, in alfalfa hay and commercial concentrate; and FB1, 560μg/kg, in commercial concentrate. The intake of mycotoxins by the horses according to the treatments are given in Table 2.

Physiological parameters

The intake of aflatoxin B_1 did not affect the respiratory rate (RR) and heart rate (HR) values of horses in the exercise test (P>0.05), however, there was a simple effect of the time of evaluation (P<0.05) (Table 3).

Blood and biochemical parameters

The inclusion of aflatoxin B_1 in the diets did not affected the blood parameters of the horses in the exercise test, except the enzyme alkaline phosphatase (ALP) (Table 4) (P<0.05). The globular volume (GV), total protein (TP), albumin (ALB) and cholesterol (COL) were evaluated at M0 through to M3 of the exercise test, and the results have been influenced by the exercise (P<0.05), with any effect from aflatoxin B_1 inclusion to the diets (P>0.05) (Table 5).

Table 2. Means of the mycotoxin intake per kg of dry matter (Kg DM) and per kg of body weight (kg BW), according to the treatments

Variables		T I (Control)		T II (50 ppb AFB1)		T III (100 ppb AFB1)	
		Kg DM	Kg BW	Kg DM	Kg BW	Kg DM	Kg BW
Mycotoxins intake (μg)	AFB1	0	0	4.6	0.1	9.4	0.2
	ZEA	241.1	6.1	246.7	5.7	249.3	5.9
	DON	1885.1	47.6	1899.1	43.9	1904.7	45.3
	FB1	195.8	4.9	205.6	4.8	210.3	5.0

Table 3. Respiratory rate and heart rate of horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Variable	Average	CV (%)	Interactions (P Value)		
			AFB_1	Time	AFB_1 X Time
Respiratory rate (mov/min)	13.3	21.5	NS	0.0000	NS
Heart rate (beat/min)	66.9	16.0	NS	0.0000	NS

CV = Coefficient of variation, NS = not significant.

The serum concentrations of urea and creatinine were evaluated at many times of the exercise test. The results showed that these parameters were not influenced by the aflatoxin B_1 inclusion in the diets (P>0.05). However, a simple effect of the exercise test was observed (Table 6).

The enzymes aspartate transferase (AST), lactate desidrogenase (LDH) and gama glutamil transferase (GGT) activities were evaluated from M0 to M9 of the exercise test, while the enzyme creatine quinase (CK) was evaluated from M0 to M7 of the exercise test. The activities of the enzymes were not affected by aflatoxin B_1 inclusion to the diets (P>0.05). However, the AST, LDH, GGT and CK activities were influenced by the exercise (P<0.05) (Table 7). The alkaline phosphatase (ALP) activity was affected by the inclusion of aflatoxin in the diets (P<0.05), presenting a higher value in horses fed diet with 100μg/kg of AFB_1 (Table 8).

Table 4. Hematological and blood biochemistry of horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Variable	Average	CV (%)	Interactions (P Value)			Reference values
			AFB_1	Time	AFB_1 X Time	
Globular Volume (%)	39.8	5.5	NS	0.0000	0.1709	38-42[A]
Total protein (gdL⁻¹)	6.2	16.8	NS	0.0003	NS	5.2-7.9[C]
Albumins (g dL⁻¹)	2.4	17.1	NS	0.0001	0.2319	2.6-3.7[C]
Cholesterol (mg dL⁻¹)	97.4	16.1	NS	0.0014	0.4034	75-150[B]
Urea (mg dL⁻¹)	33.4	10.4	NS	0.0000	0.4247	24-48[A]
Creatinine (mg dL⁻¹)	0.74	10.5	NS	0.0000	NS	1.1-1.8[A]
GGT (U L⁻¹)	7.8	38.5	NS	0.0000	0.1161	4.3-13[E]
AST (U L⁻¹)	271.3	16.3	0.0671	0.0000	0.1279	150-400[A]
CK (U L⁻¹)	323.7	27.8	0.3226	0.0000	NS	100-300[A]
LDH (U L⁻¹)	936.3	36.5	0.2882	0.000	NS	162-412[C]
ALP (U L⁻¹)	61.4	18.5	0.0227	0.3679	0.3625	100-500[D]
Glucose (mg dL⁻¹)	74.8	20.2	NS	0.0000	NS	70-140[A]
Lactate (mmol L⁻¹)	1.8	19.6	0.927	0.0000	NS	1,1-1,8[F]

[A] Rose & Hodgson (1994), [B] Robinson (1997), [C] Gonzalez & Silva (2006), [D] Kerr (2002), [E] Thomassian (2005), [F] Kaneko (2008); CV = coefficient of variation, NS = not significant.

Table 5. Mean values of Globular volume (%), Total protein (g dL⁻¹), Albumins (g dL⁻¹) and Cholesterol (mg dL⁻¹) in horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Variable	Exercise test evaluation times				CV (%)
	Pre-exercise	Immediately after the exercise test	10 min	20 min	
Globular volume (%)	34.2[D]	47.6[A]	39.9[B]	37.4[C]	5.5
Total protein (g dL⁻¹)	4.9[B]	7.0[A]	6.6[A]	6.4[A]	16.8
Albumins (g dL⁻¹)	1.9[B]	2.8[A]	2.5[A]	2.6[A]	17.1
Cholesterol (mg dL⁻¹)	81.4[B]	109.5[A]	96.7[AB]	101.8[A]	16.1

CV = Coefficient of variation; [A,B,C,D] Values followed by same letter in the line do not differ by the Tukey test (P>0.05).

Table 6. Serum concentrations of urea and creatinine (mg dL-1) in horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Variable	Exercise test evaluation times										CV%
	M0	M1	M2	M3	M4	M5	M6	M7	M8	M9	
Urea (mg dL⁻¹)	37.5[AB]	36.2[BC]	28.8[EF]	30.0[DEF]	32.1[AB]	30.2[DEF]	33.4[BCD]	37.1[AB]	42.6[A]	26.2[F]	10.4
Creatinine (mg dL⁻¹)	1.4[A]	0.7[B]	0.7[B]	0.7[B]	0.5[D]	0.6[CD]	0.7[B]	0.8[B]	0.7[BC]	0.5[D]	10.5

CV = Coefficient of variation; [A,B,C,D,E,F] Values followed by same letter in the line do not differ by the Tukey test (P>0.05).

Table 7. Activity of CK, AST, LDH, GGT (UL⁻¹) in horses fed diets with different inclusions of AFB_1 and submitted to an exercise test

Variable	Exercise test evaluation times										CV %
	M0	M1	M2	M3	M4	M5	M6	M7	M8	M9	
CK (UL⁻¹)	328.4[BC]	288.5[C]	343.3[BC]	484.2[A]	399.9[AB]	296.5[C]	308.7[BC]	139.7[D]	-	-	27.8
AST (UL⁻¹)	291.7[ABC]	326.6[A]	245.6[CD]	255.9[BCD]	268.6[ABC]	209.2[D]	292.3[ABC]	311.4[AB]	304.2[ABC]	207.5[D]	16.4
LDH (UL⁻¹)	932.0[ABC]	750.2[BC]	733.9[BC]	693.9[C]	1169.8[AB]	806.2[ABC]	938.7[ABC]	1257.4[A]	1156.5[A]	823.9[ABC]	36.5
GGT (UL⁻¹)	6.2[CD]	7.2[BCD]	7.2[BCD]	6.2[CD]	9.6[AB]	7.8[BC]	7.3[BCD]	9.7[AB]	11.8[A]	5.0[D]	38.5

CV = Coefficient of variation; [A,B,C,D] Values followed by same letter in a row do not differ by the Tukey test (P>0.05).

Table 8. Alkaline phosphatase activity (UL^{-1}) in horses fed diets with different inclusions of AFB$_1$ and submitted to an exercise test

Treatment	Exercise test evaluation times				Average	Interactions (P Value)		
	M0	M1	M2	M3		AFB$_1$	Time	AFB$_1$ time
T I (Control)	65.3	49.8	44.5	47.5	51.8^B			
T II (50 ppb AFB$_1$)	55.5	56.5	59.0	54.0	56.3^{AB}	0.0227	0.3679	0.3625
T III (100 ppb AFB$_1$)	75.8	82.5	75.0	72.0	76.3^A			

Coefficient of variation (CV) = 18,5%; ^{A,B} Values followed by same letter in a column do not differ by the Tukey test (P>0.05).

Table 9. Glucose (mg dL-1) and Lactate (mmol dL-1) concentrations in horses fed diets with different inclusions of AFB$_1$ and submitted to an exercise test

Variable	Exercise test evaluation times										CV %
	M0	M1	M2	M3	M4	M5	M5	M7	M8	M9	
Glucose (mg dL^{-1})	99.0^A	57.3^E	66.2^{DE}	67.83^{DE}	76.2^{ABCD}	63.6^{DE}	71.4^{BCDE}	89.6^{AB}	87.9^{ABC}	68.6^{CDE}	20.2
Lactate (mmol dL^{-1})	0.92^C	3.2^A	1.77^B	1.76^B	1.48^B	1.43^B	1.76^B	-	-	-	19.6

CV = Coefficient of variation; ^{A,B,C,D,E} Values followed by at same letter in a line do not differ by the Tukey test (P>0.05).

The serum concentration of glucose was evaluated from M0 to M9 of the exercise test, while the lactate concentration was evaluated from M0 to M7 of the exercise test. Serum glucose and lactate concentrations were not influenced by the inclusion of aflatoxin B$_1$ in the diets (P>0.05) (Table 9).

DISCUSSION

The AFB$_1$ was not detected in the feedstuffs of the basal diet. The AFB$_1$ produced in our laboratory and added to the commercial concentrate at concentrations of 50µg/kg and 100µg/kg, according to the treatments, which was the maximum amount intake by the horses, regardless of weight. Keller et al. (2007) observed in horse's feedstuffs concentrations of AFB$_1$ ranging from 0.01 to 99.4µg/kg. Franco et al. (2019) observed in maize food and maize-based feed from small-scale farms that median levels of total AF, total FB, ZEN, and DON were 100µg/kg, 680µg/kg, 160µg/kg, and 200µg/kg, in feed samples, respectively, and that co-occurrence of two or more mycotoxins was confirmed in 51% of feed samples.

Caloni & Cortinovis (2011) in a review of experimental AFB$_1$ intoxication in horses reported that, although inconclusive, aflatoxicosis can occur with AFB$_1$ concentrations between 0.5 and 1.0mg/kg in the diet, which induce clinical signals and hepatic damages, depending on the exposure time. The liver is a target organ in all affected animals, where the toxin induces centrilobular necrosis. Horses with aflatoxicosis show nonspecific clinical signs, such as cough, inappetence, depression, fever, tremor and ataxia. However, the blood and physiological alterations of horses in this research are resulting from the exercise test itself, with no effects from aflatoxin, except for the alkaline phosphatase activity. Dedar et al. (2019) observed growth retardation in foals, weight loss and deaths in horses ingesting millet forage contaminated with levels of 24µg of aflatoxins/kg to 70µg of aflatoxins/kg of forage. For the authors, monitoring serum/plasma GGT levels was found to be a sensitive biochemical indicator to identify liver damage caused by mycotoxins. The daily intake of horses of diet in treatment III (100µg/kg of AFB$_1$), corresponding a 9.4µg AFB$_1$ by kg DM, did not result in any clinical signs in the horses.

The simultaneous occurrence of DON, ZEA and FB$_1$ in the feedstuffs of basal diet is related to the 25 to 30% of corn in the diet. The concentration of mycotoxins in feedstuffs for horses is a serious problem, and the weather in Brazil is favorable to fungi multiplication and mycotoxins production (Keller et al. 2007, Schulz et al. 2015). Schumann et al. (2016) and Franco et al. (2019) pointed out that the simultaneous occurrence of Fusarium mycotoxins, such as DON and ZEA, is an important issue to be observed due to its implications on animal health and welfare.

The concentration of DON in the basal diet was 5.6mg/kg, with similar concentrations in alfafa hay and in the concentrate. Caloni & Cortinovis (2011) and Cortinovis et al. (2012) observed that horses fed naturally DON contaminated barley between 36 and 44mg/kg during 40 days they did not show any negative effects, such as refusing feed or alteration of blood biochemistry and hematology, suggesting that like ruminants, horses are relatively resistant to the adverse effects of DON.

The ZEA concentration in feedstuffs were 446.5µg/kg and 261.5µg/kg for concentrate and alfafa, respectively. Gimeno & Quintanilla (1983) reported natural outbreaks from feedstuffs containing approximately 2.7mg/kg of ZEA in horses. Intoxicated mares refused the diet, and presented vulvar edema, vaginal prolapse and internal hemorrhage, and severe sagging of the genitalia. In this research, no clinical symptoms were observed in the horses, suggesting that the amount of ZEA present in the basal diet was not enough to cause clinical alterations. It should be noted that the concentration of ZEA was lower than the European Union (2006) of 3mg/kg for corn by-products.

The FB$_1$ concentration in feedstuffs was 560µg/kg. Horses are the most sensitive specie to FB1 toxicity, and FB$_1$ concentrations of 20µg/kg and 120µg/kg in feed are already able to cause outbreak in this specie (Bertero et al. 2018). Ross et al. (1991) analyzed feedstuffs associated with an equine leukoencephalomalacea outbreaks observed contamination with FB$_1$ with levels ranging from 1 to 126mg/kg. Most samples presented levels above 10mg/kg. According to Reed et al. (2004) feedstuffs containing concentrations greater than

10mg/kg of fumonisin are fatal to horses. In this research, no clinical signs compatible with equine leukoencephalomalacea were observed, however the hematological and enzymatic alterations should be evaluated as they may be related to the simultaneous presence of several mycotoxins, especially aflatoxin B_1.

The physiological parameters Respiratory rate (RR) and Heart rate (HR) were evaluated at pre-exercise (M0), immediately after the exercise (M1) and at the times of 10 (M2) and 20 minutes after exercise (M3) (Table 3). There were no effects of AFB_1 inclusion in the diets in the respiratory (RR) and heart rate (HR) of the horses (P>0.05). The results showed the response of horses to exercise, with an increase of both RR and HR immediately after exercise (M1), and with a tendency to return to basal levels at 20 min (M3) after exercise. The values differed from those observed by Paludo et al. (2002) for Brazilian Sport Horses that showed mean values of heart rates of 40.8±4.7, and respiratory rate of 16.7±3.1, in the pre-exercise and of 53.7±8.2 and 37.3±11.5, for heart and respiratory rates, respectively, in the post-exercise period.

The inclusion of AFB_1 in the diets did not affected the blood parameters (Table 4), except for the alkaline phosphatase activity. The presence of aflatoxin together with other mycotoxins, especially FB, could be expected to cause changes in the blood profile during exercise due to hepatotoxicity. The intake of low doses of mycotoxins for long periods can influence the health and the performance of horses (Raymond et al. 2005, Broom 2015). Thus, hepatotoxic mycotoxins could be expected to impair hepatic metabolism, influencing the results of this research. However, the alterations observed were those resulting from the exercises on the horse, as a simple effect of time, with no effect to AFB_1 in the diets.

As expected, immediately after exercise (M1), the Globular volume presented a higher value; about 39% higher than the baseline value. The elevation of globular volume is related to spleen contraction. According to Kingston (2008) and McGowan (2008) the spleen has the capacity to store about 50% of the body's erythrocytes in horses. The release of the erythrocytes in the body occurs in response to sympathetic stimulation or increased circulation of adrenaline during exercise. The values for total protein and albumin in response to exercise were not influenced by the inclusion of AFB_1 in the diets, and only a simple effect of time was observed (P<0.05).

The increase of protein concentration immediately after exercise was observed, and its remained for 10 and 20 minutes (M3 and M4) as a consequence of blood concentration, redistribution of fluids and electrolytes from the vascular compartment to the extracellular spaces, and increased plasma viscosity (Kingston 2008). Fluid loss is related to the duration and intensity of exercise and returns to basal levels within 30 minutes of the exercise (Kowal et al. 2006). Cholesterol plasma concentration was not influenced by AFB_1 inclusion in the diets (P>0.05). The highest concentration of cholesterol immediately after exercise may be due to the increase in plasma viscosity arising from blood concentration (Kingston 2008). However, the values observed are within the reference levels for horses (Gonzalez & Silva 2006).

Plasma levels of urea and creatinine were evaluated during 48 hours post exercise (M0 to M9). As expected, the urea levels were little influenced by exercise. Snow et al. (1982) stated that the main route of energy generation used

by horses is glycolytic and anaerobic, compatible with the exercise test in the experiment: warm-up followed by three sets of gallops at a speed of 450m/min for three minutes each. Also, changes in urea concentration over time may be due to the catabolism of nitrogen from the diets. On the other hand, the serum creatinine concentrations during exercise were lower than the baseline value, which was not expected. According to Fazio et al. (2011) there is an increase in plasma creatinine concentrations during exercise due to the greater use of phosphocreatine for the generation of muscular energy. Castejón et al. (2006) observed that this increase is directly proportional to the intensity of the exercise. The values of creatinine concentration in this work were below those observed by Santiago et al. (2013) evaluating eventing horses at training, and Godoi et al. (2009) who evaluated eventing horses feeding hyperlipidemic diets. The values of urea and creatinine concentrations were below the reference values for the horses according to Rose & Hodgson (1994).

The peak of CK activity was observed at 20 minutes after exercise (M3), with a tendency to return to baseline three hours after the exercise (M5). Harris et al. (1990) reported that some animals may have high physiological enzymatic levels of CK, or even that these enzymes are removed more slowly from the circulation, or some animals may be more sensitive as to the permeability of the cellular muscular membrane compared to a similar stimulus in other animals. According to Sloet van Oldruitenborgh-Oosterbaan (1998), the plasma half-life of CK is very short, around 2 hours, which is compatible with the results observed in this experiment. The mean values of CK were higher than those observed by Oliveira et al. (2014) in Brazilian Sport Horses on treadmill exercise tests, but result of CK 12 hours after exercise (M7) was similar those found by Padilha et al. (2017) in the same breed, a 48-h rest by training.

The AST serum activity shows a standard exercise profile (Thomassian et al. 2007). The peak of AST activity was observed immediately after exercise (M1) and a tendency to increase 6 hours post exercise (M6). The high concentrations of AST immediately after exercise are related to the physiological process of fluid transfer to the extravascular space during exercise, increasing the AST activity in plasma, with a return to the 30-minute-baseline (Kingston 2008). The results of AST 48 h post exercise (M9) are very similar to those found by Padilha et al. (2017) in adult Brazilian sport horses a 48-h rest after training.

Differently from AST, the LDH activity did not present a standard profile in response to exercise, and the LDH peak was observed 12 hours post exercise (M7). The results observed were higher than the values reported by Oliveira et al. (2014) in horses on treadmill exercise tests. The large variation of LDH plasma activity can be explained by the distribution of this enzyme in several different tissues. The values of LDH plasma activity refer to the sum of several isoenzymes, so the interpretation of elevated LDH values may be compromised (Harris et al. 1990). In addition, non-pathological states may also cause increase in AST activity like LDH, as well as the collection methods, processing and equipment used for the analyzes (Sloet van Oldruitenborgh-Oosterbaan M. 1998, Thomassian et al. 2007).

The GGT activity was not influenced by the inclusion of AFB_1 in the diets (P>0.05). The increase of the enzyme is

indicative of liver damage, cholestasis and proliferation of bile ducts in all animal species (Kerr 2002, Kingston 2008, Dedar et al. 2019). Horses submitted to exercise conditioning for a prolonged period may present an increase of GGT (Balarin et al. 2005). In this experiment, the GGT activity had a peak at about 60 minutes after exercise (M4), oscillating for up to 24 hours (M8). The pre-exercise physical training period in this work during three weeks, was very short, when compared with the 12 months of training cited by Balarin et al. (2005). However, the values observed are within the basal levels for horses, ranging from 4.3 to 13UL^{-1} (Thomassian 2005).

The Alkaline phosphatase (ALP) activity was affected by the AFB_1 inclusion in the diets (P<0.05), and ALP activity increased in horses fed diet with the 100μg/kg of AFB_1. ALP is an enzyme widely distributed in the body, and the hepatic isoform predominates in the serum, and the higher values of ALP activity is indicative of liver damage in horses (Kerr 2002). Kingston (2008) reported ALP increases in horse serum due to prolonged exercise; however, it is not clear whether this increase is from bone or hepatic origin. Hank et al. (1993) observed that about 17.9% of serum ALP in mature horses is from the bones. Trueman et al. (1983) stated that increases in ALP are often unexplained and that concentrations between 0-599IU/L can be considered normal. In this work, the horses were submitted to exercise conditioning during three weeks and the ALP activity in horses fed diet with 50μg/kg of AFB_1 was higher than ALP activity in horses fed basal diet. The ALP activity in horses fed diet with 100μg/kg of AFB_1 was higher than the ALP activity in horses feeding the other two diets. All the animals were submitted the same exercise intensity, and ALP was monitored up to 20 minutes after the test (M3).

Liver damage and gastrointestinal disorders may occur in horses feeding diets containing from 2 to 50μg/kg of AFB_1 (Reed et al. 2004). Kaneko et al. (2008) reported that chronic intoxication of pigs with fumonisins is associated with increases of ALP, AST and GGT. Thus, the potential interaction and synergism of AFB_1 with FB_1 (CAST 2003) which may have influenced the ALP results and should be considered. Thus, it appears that the results of ALP activity were from hepatic tissue and its activity is directly influenced by the increased AFB_1 concentration of the diets. However, the results observed are within the clinical values considered normal for the horses, according to Trueman et al. (1983).

The serum glucose and lactate concentrations (Table 9) were not affected by the AFB_1 inclusion in the diets (P>0.05). Glucose and lactate concentrations reflect the energy metabolism and performance. The mycotoxins were expected to affect the hepatic metabolism and animal response to exercise since the liver plays a central role in metabolism, synthesizing and distributing nutrients to the peripheral organs through blood circulation (Gonzalez & Silva 2006), however, did not occur. Higher glucose concentration was observed during the rest period (pre-exercise). According to Kerr (2002), the glucose concentration increases during exercises due to the adrenaline. In this work, a decrease in glucose levels was observed, which glucose levels were restored after feeding. According to Pöso et al. (2004), changes in glucose concentration depend on the type of exercise, and may increase or decrease in short-term exercises. The serum values for glucose in this work were higher than those observed by Santiago et al. (2013) in eventing horses in the initial phase of training as observed up to the

moment M4. However, the glucose values reported here are within the serum levels of the horses (Rose & Hodgson 1994).

The peak in lactate concentration occurred immediately after exercise (M1). Lactate is an intermediate product of the metabolism of carbohydrates during anaerobic glycolysis. Under anaerobic conditions, such as in intense or prolonged physical activity, pyruvic acid is converted to lactic acid, in a reaction mediated by the Lactate dehydrogenase (Kaneko et al. 2008). The time to return lactate concentration to a baseline is a measure of the fitness of horses (Evans et al. 1995). The values observed in this study are similar to those observed by Santiago et al. (2013) working with horses that were beginners in eventing training.

CONCLUSION

The mycotoxins in the diets during 28 days doesn't influence the physiological, hematological and blood biochemical responses of the horses, except for the alkaline phosphatase activity, which presented higher activity in the diet with 100μg/kg of AFB_1, suggesting a hepatotoxic action and synergistic effect with the others mycotoxins naturally present in the feedstuffs.

Conflict of interest statement.- The authors have no competing interests.

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