

BLOOD, METABOLIC AND ENDOCRINE BIOMARKERS IN DONKEYS (*Equus africanus asinus*) SUPPLEMENTED WITH DIFFERENT ENERGY SOURCES

[Biomarcadores sanguíneos, metabólicos e endócrinos de jumentos (*Equus africanus asinus*) suplementados com diferentes fontes de energia]

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ABSTRACT – Blood and endocrine biomarkers have rarely been described in donkeys. This study aimed to analyze the effects of isoenergetic supplementation with three energy sources for 8wk on blood and endocrine biomarkers in donkeys. Fifteen donkeys were used and divided into 3 groups (extruded feed (ExF), extruded feed plus corn (C+ExF), and corn (C) and were supplemented isocalorically. Blood samples were collected at three times (pre-test, 4 and 8 weeks after supplementation). After 8wk, there were not significant difference between the treatment but detected between the phase, where results showed decreased values of red blood cells, hemoglobin, hematocrit, other cells, total plasma protein, albumin, creatinine, uric acid, GGT, AF and NEFA ($P < 0.05$) and increased values of mean corpuscular volume, mean corpuscular hemoglobin concentration, lymphocytes, urea, AST and ALT ($P < 0.05$). Analyzing postprandial glucose and insulin results, it was not observed differences among the treatments for [glucose] ($P > 0.05$), however, differences were significant for [insulin] ($P < 0.05$). ExF group exhibiting the highest insulin concentration ($P < 0.05$). As for the experimental phases, there were differences for glucose ($P < 0.05$), with the highest mean concentration observed at the end of blood collection (+ 4 h). It was concluded that supplementation with three different combination of energy source in adult donkeys improve homeostasis of the energetic biomarker, reducing NEFA concentration without changes in post-prandial glucose but modifying post-prandial insulin, and increase immunological capacity associated with an increase in the lymphocyte number.

Keywords: NEFA; Insulin; Metabolism; Nutrition; Ass.

RESUMO – Biomarcadores sanguíneos e endócrinos são pouco descritos para os jumentos. Esse estudo objetivou analisar os efeitos da suplementação isoenergética com três diferentes fontes de energia durante 8 semanas sobre os biomarcadores sanguíneos e endócrinos em jumentos. Foram utilizados 15 jumentos, divididos em três grupos (alimento extrusado (ExF), alimento extrusado e milho (ExF+C), e milho (C), suplementados isocaloricamente. Amostras de sangue foram colhidas em fases (pré-teste, e com 4 e 8 semanas após suplementação). Após 8 semanas os resultados demonstraram não haver diferenças significativas entre os tratamentos mas ocorrendo entre as fases, com redução nas hemácias, hemoglobina, hematócrito, outros células, proteínas plasmáticas totais, albumina, creatinina, ácido úrico, GGT, FA, AGNE ($P < 0,05$) e elevação do VCM, CHCM, linfócitos, ureia, AST e ALT ($P < 0,05$). Analisando-se as concentrações da glicose e insulina pós-prandial, não foram observadas diferenças entre os tratamentos para a glicose ($P < 0,05$), mas diferenças significativas para a insulina ($P > 0,05$). O grupo ExF apresentou maior concentração de insulina ($P < 05$). Analisando-se o fase experimental, foram observas diferenças na glicose ($P < 0,05$), com a maior concentração observada ao final da colheita de sangue (+ 4h). Conclui-se que a suplementação com diferentes fontes de energia melhora a homeostasis dos parâmetros energéticos, com a redução da AGNE mas sem modificar a concentração pós-prandial da glicose mas modificando a da insulina, e da melhora da capacidade imunológica, com elevação da concentração do linfócitos.

Palavras-Chave: AGNE; insulina; metabolismo; nutrição; asinino.

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INTRODUCTION

Donkeys (*Equus africanus asinus*) have been part of the agricultural around the World but, due to agricultural mechanization, the importance of these animals in different agricultural activities was reduced; consequently, the number of abandoned animals increased in some countries. This abandonment has stimulated free reproduction and gathering of these animals in sanctuaries or apprehension of these animals in animal-control facilities. In these places, the nutritional needs of donkeys may be over- or underestimated, favoring the onset of nutritional and metabolic disorders, like laminitis, as donkeys are not fed according to the recommendations for horses.

Another important issue for raising donkey is the scarce amount of information on blood and endocrine biomarker profiles when compared with horses, hampering the assessment of the effects of nutritional programs on the animals' metabolism. Moreover, some of the values described for donkeys were generated from studies in animals from different countries (Al-Busadah & Homeida, 2005; Mot et al., 2010; Simenew et al., 2011; Etena et al., 2011; Sgorbini et al., 2013), with little information available on national breeds (Oliveira et al., 1974; Girardi et al., 2014; Macedo et al., 2014).

So, there is a lack of information on the effects of different nutritional programs on blood and endocrine biomarkers in healthy donkeys in Brazil, which may compromise the welfare systems in this group of animals. To date, very few research have investigated the effects of different nutritional programs on blood and endocrine biomarkers in Brazilian donkeys. Thus, the present study had two objectives. First, we analyzed possible changes in hematological and metabolic biomarkers of Northeast donkeys subjected to isoenergetic supplementation, using concentrates with different combinations of energy sources, during 8 weeks. Second, we aimed to characterize glucose and insulin concentration curves at the end of 8 weeks of experimental period using different concentrates. It was hypothesized that supplementation with different energy sources would modify blood and endocrine biomarkers due to the effects on the overall metabolism in donkeys and produce different patterns in insulin/glucose curves for types of concentrates used in these animals' nutritional program.

MATERIAL AND METHODS

All methods used in this research were approved by Federal Rural University of Pernambuco Animal Care (CEUA#23082004420090/2015).

A total of 15 healthy donkeys of the Brazilian Northeast or Nordestino breed (Nobre, 1999) were used and they do not have hoof problems and were of both genders (male: n = 9; female: n = 6), adults, aged between 4 and 10 years, and with body masses ranging between 130 and 150 kg. All of the animals originated from different small farmers in Limoeiro, state of Pernambuco (LAT 7°52'52" Sul; LONG 35°29'40" Oeste), and were randomly divided into three groups: Extruded Feed group (ExF) (10.0% crude protein (CP), 6.0% crude fiber; 20.0% fat; Equiturbo, Guabi Animal Nutrition, Goiana-PE, Brazil); Corn grains (50% of energy) plus extruded feed (50% of energy; Equiturbo) group (C+ExF), and Corn grain group (C)(11.7% CP; 12.4% neutral detergent fiber; 4.9% fat).

The supply of feed and hay was individualized, and each group was formed with three males and two females, kept in two paddocks (~0.5 hectare/paddock) for the separation of males and females. Supplementation for the three groups was isocaloric (1.0 Mcal/day/animal), representing ~25% of the energy needs for donkeys being kept for maintenance (NRC, 2007), with the remainder of the energy obtained from forage. The animals had free access to water and mineral salt for equines (Guabiphos Centauro Cromo, Guabi Animal Nutrition, Goiana-PE, Brazil). The forage was Tifton 85 hay (*Cynodon* spp.) (~6.0 kg/day/animal), divided into three daily meals.

All animals went through a two-week adjustment period for the management system before supplementation. They were also dewormed with ivermectin (Equivan®, Merial do Brasil, São Paulo-SP, Brazil) according to their weight in the pre-trial phase. The females were not pregnant or lactating. The body masses of the donkeys were determined according to the formula previously described (Pearson & Ouassat, 2000).

Blood samples were collected at three time-points (pre-trial and 4 and 8 weeks after starting supplementation) from 12-hour fasted animals, using pre-chilled vacuum tubes containing ethylenediaminetetraacetic acid (EDTA) and sodium heparin. Samples in EDTA tubes were used to assess hematological biomarkers in semi-automatic equipment (PocH 100, Roche®, São Paulo-SP, Brazil). The heparinized samples were centrifuged to obtain plasma, which was frozen for further analysis of the following metabolic and endocrine biomarkers: total plasma proteins (TPP), albumin, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GGT); alkaline phosphatase (AP), uric acid, triglycerides, total cholesterol, non-esterified fatty acids (NEFA),

glucose and insulin. Biochemical analyses were performed in semi-automatic equipment (D-500, Doles, São Paulo-SP, Brazil) using commercial kits of the same brand, except for NEFA (NEFA Randox®, Randox Laboratories, São Paulo-SP, Brazil). For the postprandial glucose and insulin curves, samples were collected at the following time-points: pre-trial and after 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 hours. Insulin was measured through enzyme-linked immunosorbent assay (ELISA) using a commercial kit (BioAssay Systems, Hayward, CA, USA).

The results of the supplementation phases and postprandial glucose and insulin curves were analyzed by two-way ANOVA (type of concentrate and phase of the experiment or curve), with P set at 5%. Tukey's test was used as a post hoc test with a 5% P-value. The results were also subjected to the Pearson correlation method with a 1% P-value. Statistical analyses were performed with SigmaPlot® 13 software (Systat Software Inc., USA), and the results are expressed as means +/- mean standard errors.

RESULTS AND DISCUSSION

The results of the supplementation phases showed no differences among the treatments for nearly all

blood and metabolic biomarkers analyzed ($P > 0.05$). However, there were differences among the experimental phases, regardless of the experimental treatment, for several biomarkers ($P < 0.05$). Only [Urea] exhibited interactions among the treatments and phases ($P < 0.05$), where Corn group showed the highest variations after eight weeks of supplementation. Thus, the results depicted in Tables 1 and 2 are related to the comparison among the experimental phases (pre-trial and + 4 and + 8 weeks), regardless of the treatment.

As for the hematological biomarkers, at the end of eight weeks of supplementation, there were decreases in red blood count [RBC], hemoglobin [HB], hematocrit [HT] and [other cells] ($P < 0.05$), followed by increases in mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH] and [lymphocytes] ($P < 0.05$) (Table 1). No significant changes were observed for the mean corpuscular hemoglobin concentration [MCHC], red blood cell distribution width-standard deviation [RDW-SD], red blood cell distribution width-corpuscular volume [RDW-CV] and [leukocytes] in the same period (Table 1). [Platelets] were significantly increased after 4 weeks of supplementation; however, after 8 weeks, they returned the pre-trial concentrations (Table 1).

Table 1. Results of blood biomarkers in donkeys supplemented isocalorically with different types of concentrates for 8 weeks.

Hematological biomarker	Experimental phase		
	Pre-trial	After 4 weeks	After 8 weeks
Red blood cells, $\times 10^6/\text{mm}^3$	5.64 \pm 0.11 ^A	5.18 \pm 0.11 ^B	5.18 \pm 0.11 ^B
Hemoglobin, g/dL	10.67 \pm 0.20 ^A	9.96 \pm 0.20 ^B	10.08 \pm 0.20 ^{AB}
Hematocrit, %	31.38 \pm 0.58 ^A	29.05 \pm 0.58 ^B	29.45 \pm 0.58 ^{AB}
MCV, fL	55.73 \pm 0.21 ^B	56.16 \pm 0.21 ^{AB}	56.79 \pm 0.21 ^A
MCH, pg	18.98 \pm 0.08 ^B	19.26 \pm 0.08 ^{AB}	19.35 \pm 0.08 ^A
MCHC, g/L	33.37 \pm 0.40	34.28 \pm 0.40	34.07 \pm 0.40
RDW-SD, fL	42.98 \pm 0.31	42.58 \pm 0.31	43.00 \pm 0.31
RDW-CV, %	18.80 \pm 0.18	18.58 \pm 0.18	18.69 \pm 0.18
Leukocytes, $\times 10^3/\text{mm}^3$	10.13 \pm 0.39	9.25 \pm 0.39	9.19 \pm 0.39
Lymphocytes, $\times 10^3/\text{mm}^3$	3.18 \pm 0.11 ^B	3.39 \pm 0.11 ^{AB}	3.70 \pm 0.11 ^A
Other cells, $\times 10^3/\text{mm}^3$	6.95 \pm 0.30 ^A	5.86 \pm 0.30 ^B	5.25 \pm 0.30 ^B
Platelets, $\times 10^9/\text{L}$	128.13 \pm 11.54 ^B	212.40 \pm 11.54 ^A	123.40 \pm 11.54 ^B

Note: different letters in the same line indicate $P < 0.05$ by Tukey's test.

Among the metabolic biomarkers, decreases in [TPP], [albumin], [creatinine], [uric acid], gamma

glutamyl transpeptidase [GGT], alkaline phosphatase [AP] and non-esterified fatty acids

[NEFA] were observed after 8 weeks ($P < 0.05$), whereas increases in [Urea], aspartate aminotransferase [AST] and alanine aminotransferase [ALT] were observed in the same

period ($P < 0.05$) (Table 2). No significant variations were observed for [triglycerides], [total cholesterol] and body mass in the experimental period (Table 2).

Table 2. Results of metabolic biomarkers and body mass in donkeys supplemented isocalorically with different concentrates for 8 weeks.

Metabolic biomarker	Experimental phase		
	Pre-trial	After 4 weeks	After 8 weeks
Total plasma proteins, mg/dL	8.01 ± 0.18 ^A	6.90 ± 0.18 ^B	6.30 ± 0.18 ^B
Albumin, mg/dL	2.46 ± 0.20 ^A	2.36 ± 0.20 ^B	2.14 ± 0.20 ^C
Urea, mg/dL	12.67 ± 0.55 ^{AB}	11.12 ± 0.55 ^B	13.17 ± 0.55 ^A
Creatinine, mg/dL	1.07 ± 0.03 ^A	0.96 ± 0.03 ^B	0.80 ± 0.03 ^C
Uric Acid, mg/dL	0.233 ± 0.002 ^A	0.222 ± 0.002 ^B	0.221 ± 0.002 ^B
Aspartate aminotransferase, U/L	220.36 ± 6.12 ^C	246.59 ± 6.12 ^B	274.98 ± 6.12 ^A
Alanine aminotransferase, U/L	15.87 ± 0.67 ^B	19.77 ± 0.67 ^{AB}	20.95 ± 0.67 ^A
Gamma-glutamyl transpeptidase, U/L	116.88 ± 3.43 ^A	112.25 ± 3.43 ^A	82.95 ± 3.43 ^B
Alkaline phosphatase, U/L	462.54 ± 25.33 ^A	395.75 ± 25.33 ^{AB}	351.35 ± 25.33 ^B
Triglycerides, mg/dL	46.34 ± 5.88	47.03 ± 5.46	45.65 ± 5.44
Total Cholesterol, mg/dL	58.98 ± 2.60	61.13 ± 2.42	67.15 ± 2.42
Non-esterified fatty acids, mmol/L	0.71 ± 0.07 ^A	0.17 ± 0.07 ^B	0.11 ± 0.07 ^B
Body mass, kg	139.50 ± 6.70	141.00 ± 5.00	144.00 ± 6.30

Note: different letters in the same line indicate $P < 0.05$ by Tukey's test.

The results of postprandial insulin showed differences among the treatments ($P < 0.05$), whereas there were no differences in postprandial glucose ($P > 0.05$) (Figure 1). The ExF group (~5.3 μ IU/mL) showed a higher insulin concentration when compared to the C group (~2.3 μ IU/mL) ($P < 0.05$), while the insulin concentration in the C+ExF group (~3.1 μ IU/mL) was similar to the other groups ($P > 0.05$). There were also differences in glucose among the experimental phases ($P < 0.05$), with the highest mean concentration observed at the end of blood collection (+4 h) (~7.0 mg/dL) (Figure 1). There was a trend towards an interaction between phases and treatments for postprandial glucose ($P = 0.053$).

Significant correlations were also detected between concentrations of albumin and NEFA ($R = 0.48$; $P < 0.01$), TPP and NEFA ($R = 0.46$; $P < 0.01$), triglycerides and TPP ($R = 0.46$; $P < 0.01$) and triglycerides and NEFA ($R = 0.30$; $P < 0.01$).

Throughout the experiment, the climate remained stable with no rain and with a mean temperature and relative humidity of 26°C and 65%, respectively. The animals remained healthy throughout the experiment.

Hematological biomarker values reflect the effects of nutritional programs and animal health. In the present study, RBC was lower at the end of the 8wk (~8%) than at the pre-trial ($P < 0.05$). As for HB and HT, the pre-trial values were similar to those found after 8wk of supplementation ($P > 0.05$). Combined, these results support the idea that donkeys did not develop anemia, even with a decrease in RBC. It should also be noted that the values of the red blood cell components in the current experiment were slightly below or were similar to those described in the literature for other breeds and the same breed (Bossche, 1987; Mori et al., 2004; Gul et al., 2007; Gravena et al., 2010; Macedo et al., 2014).

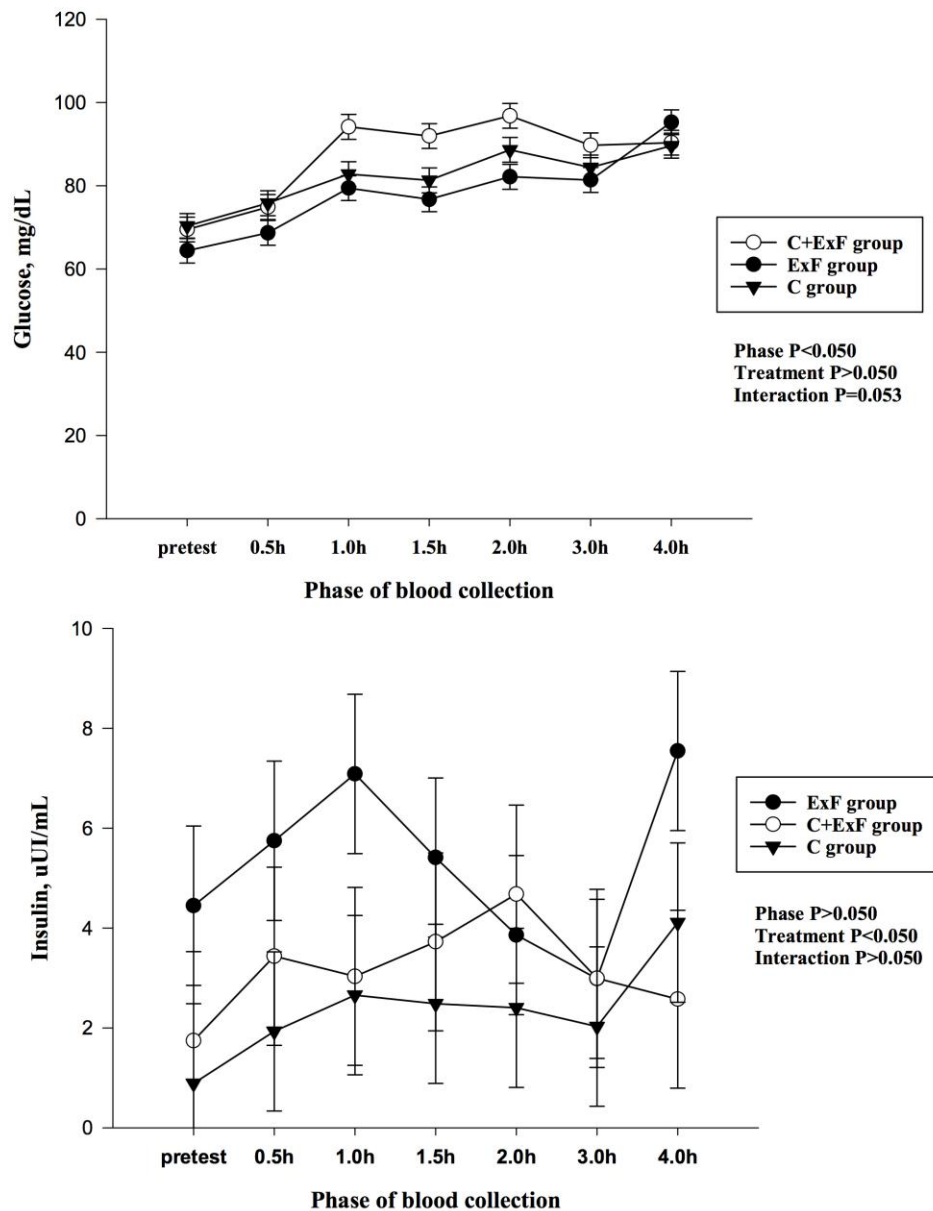


Figure 1. Postprandial glucose (a) and insulin (b) concentration curves in the donkeys supplemented isocalorically with different concentrates for 8 weeks. Notes: ExF group: extruded feed; C+ExF group: corn plus extruded feed; C group: corn feed.

Anisocytosis indices, such as RDW-SD and RDW-CV, are not yet defined for the Northeast breed but these indices, along with MCV and MCH, serve as indicators of the possible types of anemia in equines. The concentrations of RDW-SD and RDW-CV did not vary significantly and were similar to those described by Silva et al. (2014). These authors report that RDW-SD and RDW-CV concentrations in donkeys may vary from 40.0 to 43.0fL and from 18.0 to 19.0%, respectively. The MCV and MCH concentrations increased after 8wk ($P < 0.05$), reflecting a reduction in RBC and the maintenance of HT and HB. A study with German donkeys (Bossche, 1987) showed that the values of

MCV, MCH and MCHC were ~55.6fL, ~19.0pg and ~34.0g/L, respectively, similar to what was found here. In horses, RDW-SD and RDW-CV values vary widely; however, they may be as accurate as MCV and MCH to detect anemia. Nevertheless, to characterize a regenerative anemia in equines, RDW must be high and MCV must be more than 10fL above normal (Lording, 2008).

The concentrations of the leukocytes are indicative of animal health status. In the present study, leukocyte counting did not change ($P > 0.05$), remaining near $9.5 \times 10^3/\text{mm}^3$. Different publications report leukocyte concentrations in donkeys between

5.0 and $12.0 \times 10^3/\text{mm}^3$ (Bossche, 1987; Folch; Jordana; Cuenca, 1997; Mori et al., 2004; Garba et al., 2015). This wide variation may be associated with different factors (husbandry conditions, nutrition, breed, etc.); thus, these factors should be well described in the publications to allow a more thorough comparison among experiments.

Lymphocytes are important indicators of the immune responsiveness against diseases. In the present study, a significant increase (~16%) in lymphocyte count was observed ($P < 0.05$), indicating a possible improvement of the animals' immune response after 8wk of supplementation. The values for lymphocytes range from 2.0 to $5.5 \times 10^3/\text{mm}^3$ (Folch; Jordana; Cuenca, 1997; Gul et al., 2007; Gravena et al., 2010); thus, the lymphocyte values reported here before and after supplementation are within this range. Lymphocytes accounted for 42% of leukocytes at the end of 8wk, compared to 31% in the pre-trial. In horses, it is expected that 50 to 70% of white blood cells are neutrophils, less than 10% are monocytes, and up to 3% are eosinophils; basophils are rare (Carrick & Begg, 2008); however, these values have not been well defined for donkeys.

Still considering the findings on hematological biomarkers, the concentration of platelets varied significantly, showing an increase in the first four weeks (~66%) but returning to levels observed at the pre-trial after 8wk. Different studies show great variations in platelets' concentration in horses, as it may be affected by diverse factors, such as nutrition and immunological agents. In a study using Catalanian donkeys, the platelet was $\sim 236.0 \times 10^9/\text{L}$ (Jordana; Folch; Cuenca, 1998), similar to values reported for adult donkeys of the Ragusana breed ($\sim 220.0 \times 10^9/\text{L}$) (Caldin et al., 2005). Lower values were detected in eight-week-old donkeys of the Amianta breed ($\sim 180.0 \times 10^9/\text{L}$) (Sgorbini et al., 2013). By analyzing the values reported in these publications, it can be noted that the donkeys in the present study exhibited much lower values ($\sim 123.0 \times 10^9/\text{L}$); however, it is important to highlight that platelet concentrations may vary from 100.0 to $350.0 \text{ mm} \times 10^9/\text{L}$ in ponies and donkeys (Lording, 2008). Further studies should be performed to better understand platelet counts in donkeys under different situations and measured using different automatic equipment.

Protein metabolism biomarkers are used as indicators of nutritional status, especially of protein "turn-over". In the present study, total plasma protein (TPP) decreased significantly (~21%); however, it remained within the expected range (6.0-8.5mg/dL) (Bossche, 1987; Mori et al., 2004; Simenew et al., 2011; Girardi et al., 2014). Significant decreases were also detected for

albumin (~13%), creatinine (~25%) and uric acid (~5%) concentrations; however, the urea concentration at the end of 8wk did not differ from pre-trial values. The albumin concentration in Northeast breed was higher than that described by Bossche (1987), who reported an albumin concentration of ~25% of TPP. It is important to remember that albumin is an important transporter of NEFA (Brinkmann; Gerken; Riek, 2013); thus, in the present study, a decrease in this biomarker may be associated with a decrease in NEFA.

Different factors may be associated with decreases in protein biomarkers, such as an increase in the plasma volume and the time to adapt to the new nutritional program. In the present study, plasma volume was not assessed; however, the decrease in RBCs without any changes in HT may be associated with a discrete change in the plasma volume. In the second aspect, it is known that equines require a few weeks to adapt their digestive processes to a new feed. As the experiment lasted for 8wk, perhaps the animals were not fully adapted at the end of this period, consequently not favoring the maintenance of or increases in TPP and albumin concentrations.

Liver enzymes reflect the activity of the liver tissue and may be important to evaluate new nutritional programs. In the present study, AST and ALT levels increased and GGT and AP levels decreased ($P < 0.05$), without the animals presenting any type of disorder; however, it is known that the values of these enzymes vary considerably in the literature. In international studies, the concentrations of these enzymes have been described for Catalanian (AST: $\sim 255 \text{ IU/L}$; GGT: $\sim 48.0 \text{ IU/L}$) (Jordana, Folch; Cuenca, 1998) and Pakistani (AST: $\sim 245.0 \text{ IU/L}$; ALT: $\sim 27.0 \text{ IU/L}$; FA: $\sim 509.0 \text{ IU/L}$) (Gul et al., 2007) donkeys. In Brazil, values are reported for the Brasileira (AST $\sim 296.0 \text{ IU/L}$, GGT $\sim 46.0 \text{ IU/L}$) (Mori et al., 2004) and Pêga (AST $\sim 274.0 \text{ IU/L}$, ALT $\sim 16.0 \text{ IU/L}$, GGT $\sim 54.0 \text{ IU/L}$, FA $\sim 183.0 \text{ IU/L}$) (Girardi et al., 2014).

Upon analyses of the aforementioned values described by different authors, it is observed that the results for the enzymes found in the present study are within these variations. In healthy horses, the concentrations of AST, GGT and AP are expected to be lower than 300, 40 and 350 IU/L, respectively (Johnston, 1986). In the present study, only GGT was above this threshold after 8wk of supplementation. These enzymes are associated with liver diseases; however, no signs of liver disorders, either acute or chronic, were observed in the animals.

Donkeys have a unique regulation of lipid metabolism biomarkers and are frequently afflicted

with lipidemia (McKenzie III, 2011). Several studies indicate that the concentrations of triglycerides (TRYG) and total cholesterol (TCHOL) range between 60.0 and 90.0mg/dL and between 67.0 and 95.0mg/dL, respectively (Jordana; Folch; Cuenca, 1998; Mori et al., 2004; Girardi et al., 2014). In Northeast donkeys, after 8wk of supplementation, the concentrations of TRYG and TCHOL were ~46.0mg/dL and ~67.0mg/dL, respectively, indicating that they had concentrations below or at the borderline of the values described above. A recent study reports the concentrations of different hormones and metabolites of energy metabolism for donkeys, showing an association between accumulations of fat in the neck and high concentrations of TRYG and leptin (Mendoza et al., 2015). In the present study, Northeast donkeys exhibited body conditions that indicated values just below fat accumulation, which may be a result of low TRYG concentrations.

Another important biomarker of fat metabolism is NEFA. This biomarker increases in equines when they are supplemented with diets rich in oil, after exercise or catabolic state; however, the variations in the NEFA concentration in the blood of these animals may be high (Jansson & Lindberg, 2012; Pagan et al., 2002; Sloet van Olddruitenborgh-Oosterbaan et al., 2002). Although there are few studies of this biomarker in donkeys and mules, anorexia or food restriction causes increases in the concentrations of TRYG and NEFA in these animals, similar to what was observed in ponies under food restriction (Brinkmann; Gerken; Riek, 2013). Also they observed an increase in NEFA in animals under food restriction, suggesting an adaptation to survive under harsh conditions, enabling them to maintain glucose levels within the normal range (Brinkmann; Gerken; Riek, 2013).

In the present study, a significant reduction in NEFA concentration was observed at the end of the 8wk (~84%), indicating large intake of energetic nutrients (glucose, volatile fatty acids and fats) in the three experimental groups, favoring the adaptation of animals to the increased availability of food. In horses, the NEFA concentration is high when they are fasting (~0.13mmol/L); however, it decreases in the postprandial period after hay ingestion (~0.05mmol/L) (Gomaa; Koeller; Schusser, 2009). Recently, Chiofalo et al. (2012) evaluated NEFA concentrations in donkeys fed with hay and oats and noted that [NEFA] rises with aging, from ~0.17mmol/L to ~0.22mmol/L, and is higher in males (~0.26mmol/L) than in females (~0.11mmol/L). These values are lower than those found in Nordestino donkeys at the pre-trial but are similar to those observed after 8wk of supplementation, indicating that supplementation

favoured the regulation of energy metabolism in the experimental animals. It should also be noted that the NEFA concentration may be lower when the animal exhibits a reduction in their albumin concentration (Brinkmann; Gerken; Riek, 2013). A positive correlation was observed between albumin and NEFA ($R = 0.48$; $P < 0.01$) in this research.

The variations in glucose and insulin concentrations in donkeys resemble the values reported for healthy horses (Mendoza et al., 2015; de Laat; McGreed; Sillence, 2015); however, these analyses should take into account that a single blood sample may not characterize the metabolism of these biomarkers (Ralston, 2002). Thus, the analysis of both glucose and insulin curves is important to understand energy metabolism in horses. The concentration of glucose has been described in donkeys of the Brasileira (~59.0mg/dL) and Pêga (~70.0mg/dL) breeds and in a German breed (~85.0mg/dL) (Bossche, 1987; Mori et al., 2004; Girardi et al., 2014); however, insulin values were not reported. Recently, it was shown that glucose and insulin concentrations in 12-hour fasting donkeys were ~77.4mg/dL and 10.1 μ IU/mL, respectively (Mendoza et al., 2015).

Maintaining a consistent glucose concentration is important, and a significant decrease or increase in its levels may cause changes in lipid metabolism in equines (McKenzie III, 2011; Brinkmann; Gerken; Riek, 2013). Ralston (2002) observed that the peaks of these biomarkers (glucose: 100-200mg/dL; insulin: 190 μ IU/mL) occur 2 to 3 hours after food ingestion in horses. Differently from horses, in the present study, the peak of glucose (~90mg/dL) occurred at the end of the curve (+4 h); however, no differences among the treatments were observed ($P > 0.05$). This difference on glucose's peak between horses and donkeys need be more evaluated because have more implication in the animal nutrition and health.

In contrast, there were differences among the treatments in regard to insulin concentration ($P < 0.05$), with a peak occurring at the end of the curve (+4 h) in two groups (ExF and C). In the C+ExF group, the insulin peak occurred at the second hour of the postprandial period, like is expected in healthy horses, which were submitted to grain challenge. These differences were not expected, but recently de Laat, McGreed & Sillence (2015) commented that glucose and insulin regulation in horses have significative association with the "foregut theory", where distinct types of food may cause different levels of incretin's release, and this process has relationship this the anticipation or retardation in insulin release. In the current study we did not measure the incretins but we used three combination of food and caused different responses

for insulin concentration, which was not expected. This process may have relationship with that incretin stimulation in donkeys, like GLP-1 and GIP in horses, but this proposed process need more investigation in donkeys. The differences in the insulin concentration among the treatments may be important from a metabolic point of view for this species and thus represents an issue that should be addressed in further studies aimed at indicating more appropriate types of food for donkeys.

CONCLUSION

It was concluded that supplementation with three different combination of energy source in adult donkeys improve homeostasis of the energetic biomarker, reducing NEFA concentration without changes in post-prandial glucose but modifying post-prandial insulin, and increase immunological capacity associated with an increase in the lymphocyte number. Also, it was not possible to observe significant variations in blood biomarkers among the treatments; however, significant differences were observed among the experimental phases, which is important because all three combinations may be used for donkeys for a short period.

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