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Effects of 48-hour feed deprivation on acute-phase response in horses

Efeitos da privação alimentar de 48 horas na resposta de fase aguda em cavalos

Paula Alessandra Di Filippo[1*], Bárbara Ribeiro Duarte[1], Antônio Peixoto Albernaz[1], Leandro Abreu da Fonseca[2], Inácio Silva Viana[3], Célia Raquel Quirino[1]

[1]Universidade Estadual do Norte Fluminense "Darcy Ribeiro" (UENF), Campos dos Goytacazes, Rio de Janeiro, Brazil
[2]Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil
[3]Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Jaboticabal, São Paulo, Brazil
[*]Correspondent: pdf@uenf.br

Abstract
The objective of this study was to evaluate the effect of feed restriction on acute-phase response in horses. Twenty horses were deprived of food for 48 h and others 12 animals (control) had free access to water and hay. They were closely monitored and examined, and blood samples were taken at the beginning (0) of the study and 6, 12, 18, 24, 30, 36, 42 and 48 hours afterward. Data were submitted to two-way analysis of variance with repeated measures and statistical significance was $P \leq 0.05$. The horses tolerated feed restriction without serious clinical complications. Feed restriction induced an increase in the acute-phase response by elevating serum concentrations of α_2-macroglobulin (24-38 h), ceruloplasmin (36-48 h), α_{1}-antitrypsin (30-48 h), α_{1}-acid glycoprotein (42-48 h) and haptoglobin (42-48 h). Nutrient deprivation raised the levels of circulating cortisol, which acts on the innate immune system, which then induces the acute-phase response. In conclusion, food restriction is a physical stressor for horses, capable of inducing an acute-phase protein reaction, characterized by increased production of α2-macroglobulin, ceruloplasmin, α1-antitrypsin, α1-acid glycoprotein and haptoglobin.
Keywords: Equine; Feed restriction; Inflammation; Immune response; Proteins; Stress.

Resumo
O objetivo deste estudo foi avaliar o efeito da restrição alimentar na resposta de fase aguda em equinos. Vinte cavalos foram submetidos à restrição alimentar por 48 h enquanto outros 12 animais (controle) tiveram livre acesso à água e alimento. Os animais foram monitorados, examinados e amostras de sangue foram coletadas no início (0) do estudo e com 6, 12, 18, 24, 30, 36, 42 e 48 horas de restrição alimentar. Os dados foram submetidos à análise de variância bidirecional com medidas repetidas e a significância estatística foi $P \leq 0,05$. Os cavalos toleraram a restrição alimentar sem complicações clínicas relevantes. A restrição alimentar induziu uma resposta de fase aguda caracterizada pela elevação das concentrações séricas de α2-macroglobulina (24-38 h), ceruloplasmina (36-48 h), α1-antitripsina (30-48 h), α1-glicoproteína ácida (42-48 h) e haptoglobina (42-48 h). A privação de nutrientes eleva os níveis de cortisol circulante, que atua no sistema imunológico inato o qual, então induz a resposta de fase aguda. Em conclusão, a restrição alimentar é um fator estressor físico para equinos, capaz de induzir uma reação proteica de fase aguda, caracterizada pelo aumento na produção de α2-macroglobulina, ceruloplasmina, α1-antitripsina, α1-glicoproteína ácida e haptoglobina.
Palavras-chave: Equinos; Restrição alimentar; Inflamação; Resposta imune; Proteína; Estresse.

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Introduction

Equines are inevitably exposed to stress during their life, including psychological, physiological and physical stressors associated with routine management practices[1]. An example is feed restriction. There are various situations in horse breeding where feed intake is suboptimal, either due to feed scarcity (e.g., severe weather, physical confinement, transportation, drought, overcrowding, competitions, exercise) or resulting from an adaptive response (e.g., fear, social isolation, surgery, disease, change of routine or living conditions)[2]. The decrease in energy and nutrient intake can affect performance and host immunological defense. These stress-induced immune responses can elicit acute-phase response (APR). In cattle, feed and water deprivation can also disturb the ruminal flora and cause microbial death, resulting in the release of microbial endotoxins, which can also activate the immune system and cause an acute-phase response[3].

The systemic APR is a non-specific component of the innate immune response. It is the systemic reaction to local or systemic disturbances caused by trauma, infection, surgery, neoplasia, inflammation or stress, the goal of which is reestablishment of homeostasis and healing[4,5]. Within the first few hours of APR, protein synthesis in the liver and hepatocyte secretion are drastically altered, and there are measurable changes in the serum concentration of several plasma proteins, referred to as acute-phase proteins (APPs)[5]. In this context, feed deprivation in horse breeding imposes some degree of stress on the animals, and adversely affects their well-being. The serum levels of acute phase proteins are potential physiological indicators of stress caused by feed deprivation in cattle[4,5]. However, it has not been determined whether this also occurs in horses, and if so, how long it would take for a measurable APP response to stressors to occur in horses.

The present study was designed to determine the acute-phase response (APR) in horses submitted to feed deprivation for 48 hours. We hypothesized that feed deprivation would elicit APP reactions within 48 h, probably associated with inappetence stress.

Material and methods

Animals and housing

This study design was in accordance with the Brazilian animal ethics regulations and was approved by the ethics committee of Norte Fluminense Darcy Ribeiro State University (protocol number 384). Thirty-two mixed-breed geldings were studied (age 6.4 ± 2.0 years, weight 404.19 ± 46.93 kg). The animals belonged to a single farm and the owner consented to their use in this study. The initial body condition score (BCS) ranged from 3.0 to 4.0 points, where the BCS scale varies from 0 = emaciated to 5 = obese[6]. Horses underwent a physical examination prior to inclusion in the study and were considered clinically normal. All horses had been regularly dewormed and none had been receiving any other medication for at least the past 4 weeks. The horses' diet consisted of *ad libitum* hay at the start of the study.

Experimental design

The animals were allocated into a control group (12 animals) and feed restricted group (FR group = 20 animals), with similar mean BCS, body weight and age of both groups. The two groups were housed separately in two identical paddocks under natural light in an open outdoor shelter. The paddock had a concrete floor with no vegetation. The study was conducted in the summer, during which the mean minimum temperature was 24.2 ± 0.4 ^{0}C and maximum was 32.6 ± 0.6 ^{0}C, with average relative humidity of 75.0 ± 1.8%. The animals were normally kept in this paddock year-round with hay and trace mineralized salt *ad libitum*. During the experimental period, the control group (CT) had free access to water and hay, while the restricted group (FR) only had free access to water.

Samples and laboratory analyses

Blood was first collected (T0) from the right external jugular vein with a 12G catheter after a 7-day acclimatization period, in the morning about 4 hours after receiving hay. Additional blood samples were collected 6, 12, 18, 24, 30, 36, 42 and 48 hours later. The samples were drawn into a 10 ml syringe and then transferred to collection tubes without anticoagulant. The samples were centrifuged (4380 *g*) and the serum was placed in Eppendorf tubes, which were stored at -20 °C for 7 days until biochemical analysis.

Total proteins were determined by the Biuret[7] method (Labquest, CELM - E-225-D, BR). The serum and peritoneal acute-phase proteins were measured by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis[8], according to the manufacturer's instructions. Molecular weights and concentrations of protein fractions were determined by computed videodensitometry (C-9000, Shimadzu Corp., Kyoto, Japan). Reference markers (Sigma Chemical Co., St Louis, USA) were used to characterize proteins, with molecular weights of 29, 45, 66, 97.4, 116, and 205 kDa. Also, electrophoretic migration of proteins was compared with that of pure proteins, including albumin, transferrin, haptoglobin, ceruloplasmin, IgA, IgG, α1-antitripsin and acidic glycoprotein. All samples were analyzed in the same gel.

Statistical analysis

Data were expressed as mean ± SD and statistical significance was set at $P \leq 0.05$ for all analyses. Data collected without fasting were compared with data generated during feed deprivation. Two-way ANOVA with repeated measures, with time and feeding status as factors,

was used. The Tukey test was applied for post hoc comparison. All statistical computations were performed using SAS[9].

Results

No serious clinical and behavioral changes were observed when the horses were deprived of food, and although the feed-restricted horses were more lethargic, they remained alert and interested in their surroundings. Feed restriction induced an increase in the acute-phase response in horses by elevating serum concentrations of α_2-macroglobulin (24-38 h), ceruloplasmin (36-48 h), α_1-antitrypsin (30-48 h), α_1-acid glycoprotein (42-48 h) and haptoglobin (42-48 h) (Tables 1 and 2). However, feed restriction did not affect the concentrations of the total serum protein, transferrin (Tf), immunoglobulin G heavy chains (IgG-H), immunoglobulin G light chains (IgG-L), and apolipoprotein (Apo).

Table 1. Effects of feed restriction on the total serum protein, α_2-macroglobulin (α2M), ceruloplasmin (CP), transferrin (Tf) and α_1-antitrypsin (α_1-AT) in horses

Parameter		Hours of feed restriction								
		0	6	12	18	24	30	36	42	48
Total Protein (g L^{-1})	CT	7.54^{Aa}±0.45	7.61^{Aa}±0.47	7.70^{Aa}±0.59	7.75^{Aa}±0.56	7.82^{Aa}±0.80	7.87^{Aa}±1.25	7.88^{Aa}±0.89	8.13^{Aa}±0.77	8.20^{Aa}±0.93
	FR	7.32^{Aa}±0.5	7.40^{Aa}±0.93	7.60^{Aa}±0.95	7.53^{Aa}±1.07	7.60^{Aa}±0.70	7.73^{Aa}±0.88	7.88^{Aa}±1.16	8.26^{Aa}±1.28	8.06^{Aa}±1.31
α2M (mg/dl)	CT	241.2^{Aa}±94.4	237.2^{Aa}±86.6	244.9^{Aa}±113.2	225.0^{Aa}±97.8	237.1^{Aa}±87.5	261.3^{Aa}±137.8	224.1^{Aa}±68.4	277.2^{Aa}±133.5	206.7^{Aa}±133.5
	FR	183.6^{Aa}±100.5	238.7^{Aa}±182.4	220.5^{Aa}±104.2	244.9^{Aa}±146.8	287.0^{Aa}±175.0	296.6^{Aa}±197.2	322.0^{Aa}±225.2	351.4^{Aa}±200.4	262.1^{Aa}±212.6
CP (mg/dl)	CT	110.0^{Aa}±38.3	117.2^{Aa}±43.0	128.3^{Aa}±54.3	110.0^{Aa}±59.9	115.7^{Aa}±55.3	117.3^{Aa}±72.4	125.0^{Ba}±103.2	108.0^{Ba}±76.1	102.4^{Ba}±56.0
	FR	99.8^{Aa}±49.3	112.7^{Aa}±71.2	107.9^{Aa}±64.0	109.6^{Aa}±50.2	121.4^{Aa}±71.0	123.7^{Aa}±68.9	186.0^{Aa}±81.9	183.5^{Aa}±69.2	172.7^{Aa}±93.7
Tf (mg/dl)	CT	611.9^{Aa}±221.1	685.3^{Aa}±277.6	639.3^{Aa}±219.9	595.3^{Aa}±229.1	678.9^{Aa}±194.0	613.8^{Aa}±265.6	636.9^{Aa}±331.5	702.2^{Aa}±289.8	667.2^{Aa}±329.6
	FR	588.9^{Aa}±187.1	672.2^{Aa}±251.3	579.1^{Aa}±228.4	620.5^{Aa}±278.1	670.9^{Aa}±243.0	585.8^{Aa}±258.3	659.2^{Aa}±272.5	719.4^{Aa}±214.2	721.6^{Aa}±234.2
α1-AT (mg/dl)	CT	245.0^{Aa}±176.2	204.6^{Aa}±72.8	274.1^{Aa}±214.7	222.9^{Aa}±149.9	224.0^{Aa}±92.1	213.6^{Ba}±105.7	193.2^{Ba}±34.6	158.7^{Ba}±102.8	209.1^{Ba}±77.6
	FR	237.3^{Aa}±82.2	274.7^{Aa}±85.5	256.3^{Aa}±76.2	301.4^{Aa}±232.1	226.9^{Aa}±79.3	331.7^{Aa}±72.9	325.3^{Aa}±69.6	284.3^{Aa}±76.0	397.9^{Aa}±108.2

CT = control group; FR = feed restriction group. Means followed by different uppercase letters in the same column indicate significant differences between groups by the Tukey test at 5% probability. Means followed by different lowercase letters in the same row indicate significant differences between times by the Tukey test at 5% probability.

Table 2. Effects of feed restriction on the serum concentration of immunoglobulin G heavy chains (IgG-H), immunoglobulin G light chains (IgG-L), α_1-acid glycoprotein (AGP), haptoglobin (Hp) and apolipoprotein (Apo) in horses

Parameter		Hours of feed restriction								
		0	6	12	18	24	30	36	42	48
IgG-H (mg/dl)	CT	957.9^{Aa}±421.1	973.9^{Aa}±511.6	877.1^{Aa}±389.9	896.3^{Aa}±508.2	935.6^{Aa}±284.1	965.8^{Aa}±505.2	884.1^{Aa}±331.6	1109.3^{Aa}±533.9	941.4^{Aa}±397.9
	FR	805.4^{Aa}±396.7	940.9^{Aa}±395.9	957.8^{Aa}±406.3	877.2^{Aa}±457.9	931.3^{Aa}±357.5	916.3^{Aa}±526.9	843.8^{Aa}±374.4	1004.2^{Aa}±395.3	898.7^{Aa}±403.7
IgG-L (mg/dl)	CT	1159^{Aa}±477.7	1062^{Aa}±529.2	971^{Aa}±503.7	1052^{Aa}±388.1	1052^{Aa}±324.7	1300^{Aa}±507.1	1215^{Aa}±502.5	1201^{Aa}±434.2	1158^{Aa}±487.6
	FR	1263^{Aa}±544.1	1317^{Aa}±477.0	1198^{Aa}±546.1	1188^{Aa}±557.1	1194^{Aa}±406.0	1164^{Aa}±466.5	1080^{Aa}±261.8	1158^{Aa}±521.7	1241^{Aa}±549.7
AGP (mg/dl)	CT	161.0^{Aa}±148.2	136.8^{Aa}±76.1	145.4^{Aa}±60.4	162.3^{Aa}±137.6	147.5^{Aa}±94.6	156.7^{Aa}±106.9	137.1^{Aa}±109.9	150.9^{Ba}±127.5	136.1^{Ba}±111.8
	FR	140.3^{Aa}±46.1	149.2^{Aa}±82.7	142.2^{Aa}±83.8	162.1^{Aa}±87.6	160.2^{Aa}±86.9	164.6^{Aa}±109.4	155.8^{Aa}±83.6	209.9^{Aa}±110.1	283.6^{Aa}±95.5
Hp(mg/dl)	CT	300.2^{Aa}±180.4	291.4^{Aa}±144.9	327.3^{Aa}±190.8	285.4^{Aa}±146.9	325.2^{Aa}±176.8	343.1^{Aa}±199.1	352.7^{Aa}±206.4	342.3^{Ba}±221.0	336.9^{Ba}±182.3
	FR	286.3^{Aa}±114.72	307.9^{Aa}±142.9	328.9^{Aa}±207.6	319.1^{Aa}±169.4	337.6^{Aa}±166.3	319.4^{Aa}±146.0	327.6^{Aa}±148.8	484.7^{Aa}±156.5	450.4^{Aa}±169.0
Apo (mg/dl)	CT	589.9^{Aa}±206.5	556.9^{Aa}±296.0	513.0^{Aa}±61.2	487.7^{Aa}±102.2	465.9^{Aa}±81.72	552.3^{Aa}±188.1	590.6^{Aa}±235.4	534.1^{Aa}±147.6	552.6^{Aa}±125.1
	FR	509.9^{Aa}±253.4	512.8^{Aa}±150.4	480.5^{Aa}±183.0	531.8^{Aa}±203.6	523.4^{Aa}±169.4	565.8^{Aa}±187.9	500.5^{Aa}±167.6	539.0^{Aa}±197.2	550.7^{Aa}±198.9

CT = control group; FR = feed restriction group. Means followed by different uppercase letters in the same column indicate significant differences between groups by the Tukey test at 5% probability. Means followed by different lowercase letters in the same row indicate significant differences between times by the Tukey test at 5% probability.

Discussion

All animals submitted to 48-hour feed restriction became more lethargic compared to the control animals but did not present severe behavioral changes. Animals in general have a subtle mechanism that enables them to cope with environmental stimuli and maintain homeostasis. Many environmental factors can cause stress, classified as psychological stress, physical stress, and a combination of both[10]. When animals are transported, handled, mixed and/or put in isolation, they suffer psychological stress[11, 12, 13]. Extreme temperatures or food shortages are examples of physical stress and directly induce stress responses in the body. Some stressors, such as noise, pain, restraint, and weaning, are both psychological and physical[14]. Stress in animals has important economic implications and is recognized as a health problem[15]. The immune response is one of the mechanisms through which animals defend themselves against environmental challenges[10]. The acute-phase response is a non-specific component of innate immunity capable of identifying these stressful situations in animals[3].

In this study, we found that feed restriction influenced the acute-phase response. The horses showed significantly elevated concentrations of α_2-macroglobulin, ceruloplasmin, α_1-antitrypsin, α_1-acid glycoprotein and haptoglobin. Previous studies have also investigated changes in acute-phase response as potential biomarkers of stress. Kim et al.[10] reported that weaning (including milk restriction) caused significant increases in the neutrophil: lymphocyte (LY) ratio, with a significant reduction of LY. In this same study, the concentration of haptoglobin and serum amyloid A also increased significantly, as well increases in the levels of serum tumor necrosis factor-α and cortisol. That study also revealed a significant decrease in interferon-γ levels compared to the values obtained before weaning. Weaning also significantly decreased the percentage of CD25+ T cells in the peripheral blood. According to the authors, the stress, psychological and physical, of weaning can induce an acute phase response possibly through elevation of cortisol production and inflammation modulation cytokines.

Feed and water deprivation during long transportation periods (24 h) was also found to contribute to the acute-phase response and negatively affect the performance of beef cattle[17]. For the authors, feed and water restriction were major causes of the acute phase reaction and reduced the feedlot receiving performance typically detected in transported feeder cattle[17]. According to Marques et al.[18], food deprivation for 24 h with regular access to water is the main source of a neuroendocrine and acute phase reaction, including elevated circulating cortisol, NEFA and ceruloplasmin concentration in growing beef heifers. In rats, a study revealed that malnutrition induced IL-6 and a-2 macroglobulin expression in these animals[19].

Elevation in glucocorticoids during nutrient deprivation has been suggested as a possible mechanism to induce proinflammatory cytokines and the stress-induced APP response in cattle[4, 20]. Conversely, the specific effects of food restriction on the acute-phase response and acute phase proteins have not yet been determined in horses.

Additionally, nutrient deprivation events can also disturb the ruminal flora and cause microbial death, resulting in the release of microbial endotoxins, which in turn can elicit an acute-phase response[3]. It is believed that acute-phase proteins are the best markers of gut luminal content infiltration, and these proteins produced in the liver as a secondary response to toxic stimuli have been widely used as indicators of systemic inflammation[21]. After feed and water deprivation, microbial ruminal concentration takes 72 h to return to its initial levels[22], decreasing ruminal fermentative capacity and cattle feed intake[23, 24]. In a study with 28 Holstein cows, feed restriction to 40% of *ad libitum* intake for 5 days negatively affected intestinal architecture, characterized particularly by reduced ileum villus height and crypt depth[25]. There are various situations in horse breeding where feed intake is suboptimal. Besides the stress-induced APP response, these situations can have important intestinal implications in these animals.

Corroborating the findings of acute phase protein described, previously published findings revealed that during fasting, there was a reduction in the leukocyte response. There was also a decrease in gastrointestinal sounds compared to the control horses. However, fasting had no effect on body mass and body condition score, heart rate, respiratory rate, capillary filling time and body temperature[26]. Severe ulceration of the gastric squamous epithelial mucosa, caused by excess acidity, can develop rapidly in horses deprived of feed or not consuming feed[16]. In the aforementioned study, gastric ulceration was induced in horses by alternating 24-hour periods of feed deprivation and *ad libitum* access to hay, for a total of 96 hours' feed deprivation[16]. However, in our study, no clinical signs compatible with the development of gastric ulcers were observed. Nevertheless, gastroscopic examination would be the most advisable technique for this diagnosis. In any event, the animals were subjected to a shorter period of food restriction (48 hours).

Conclusion

The results obtained in the present study suggest that the food restriction for 48 hours in the horses studied induced an acute-phase protein reaction, characterized by increased production of α2-macroglobulin, ceruloplasmin, α1-antitrypsin, α1-acid glycoprotein and

haptoglobin. These findings are due to stress and cortisol release. The stress triggered by food restriction likely has important implications for the health of horses.

Conflict of interests

The authors declare they have no competing interests.

Author contributions

Formal analysis: P. A. Di Filippo, I. S. Viana, A. P. Albernaz, B. R. Duarte, L. A. Fonseca and C. R. Quirinus. *Methodology:* P.A. Di Filippo and B.R. Duarte. *Supervision:* P.A. Di Filippo. *Acquisition of financing*: P. A. Di Filippo. *Writting (original draft, review & editing)*: P. A. Di Filippo e I. S. Viana.

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