

Influence of Glutamine and Glutamate Supplementation in the Blood Levels of Horses

Helena Emília Cavalcanti da Costa Cordeiro Manso, Deyvson Ferreira de Oliveira,
Monica Miranda Hunka & Hélio Cordeiro Manso Filho

ABSTRACT

Background: The most abundant free amino acid in mammals is glutamine (GLN). Little research has focused on GLN supplementation for horses, but GLN levels in this species are known to decline after exercise and during lactation. Under physiological conditions, the body produces Gln in sufficient quantities for general metabolism, and a small part of this amino acid comes from dietary protein. Little research has so far focused on equine dietary supplementation with free glutamine or combined with other amino acids during catabolic states or in highly stressful situations. This research was conducted to evaluate the effects of equine dietary supplementation using a combination of glutamine and glutamate.

Materials, Methods & Results: The study involved four Arabian mares, not in training (~380 kg; ~12 years old) and four treatments (control, and inclusions of 1, 2 and 4% of GLN+GLU) in a Latin square model. A 7-day washout period was established between each phase. Fifty percent of the mares' maintenance energy requirements came from concentrate and 50% from hay and grazing. The other 50% came from Tifton hay (*Cynodon dactylon*), which was supplied *ad libitum*. After 7 weeks of nutritional supplementation (once a day, in the morning). In the experimental model, the mares were distributed in a Latin square design comprised of four treatments: control (without inclusion) and inclusions of 1%, 2% and 4% of supplement (AminoGut®, Ajinomoto do Brazil), and four animals. Blood was collected in five stages (fasting, and 60, 120, 240 and 360 min after feeding) in each treatment. The blood samples were analyzed to determine GLN, GLU, urea, creatinine, uric acid, total plasma protein, hematocrit and glucose levels. Glutamine and Glutamate concentrations were analyzed using the enzymatic spectrophotometric method. The results were analyzed statistically using one- and two-way ANOVA and Tukey's test with *P* set at 5%. The results indicated that GLN differed in both the group ($P < 0.001$) and between the phases of supplementation ($P < 0.001$), but no interaction occurred between them ($P > 0.05$). Significant changes in GLN levels were also observed in the 4% inclusion treatment compared to all the treatments in the fasting phase and in the + 60 min and +240 min phases of the control group ($P < 0.05$). All the other biomarkers analyzed here were unchanged (Glutamate, Urea, Creatinine, Urea, TPP, Glucose and Hematocrit) ($P > 0.05$) during the period under analysis, and remained within the normal range for the species in their current stabling conditions. The mares presented no clinical problems nor did they change their feeding behavior during the supplementation period or on the days blood was collected.

Discussion: Glutamine metabolism in horses has yet to be extensively studied. However, it has been shown that, when supplied to horses in its free form, this amino acid causes Gln levels to rise rapidly within the first 90 min of the postprandial period. This indicates that an extra amount of this amino acid may increase Gln blood levels despite intense degradation of enterocytes. This study found that supplementation with a combination of Gln+Glu can increase blood Gln levels after 6 h in the treatment involving 4% Gln+Glu included in the concentrate ($P < 0.05$). In conclusion, supplementation with GLN+GLU raised the mares' GLN levels after 360 min when 4% of GLN+GLU was included in their diet. These results may be used to establish GLN supplementation models for horses.

Keywords: amino acid, biomarkers, catabolism, equine nutrition, horses, protein metabolism.

INTRODUCTION

Glutamine (Gln) is the most abundant free amino acid in animal tissues. Under physiological conditions, different animal tissues, particularly muscle tissue, produce Gln in sufficient quantities for general metabolism, and a small part of this amino acid comes from dietary protein. Little research has so far focused on equine dietary supplementation with free glutamine or Gln combined with other amino acids during catabolic states or in highly stressful situations.

Various tissues are typically consumers of Gln, including enterocytes, colonocytes, and cells of the immune system. The small intestine plays an important role in the catabolism of circulating Gln and dietary amino acids, and most Gln and almost all Glutamate (Glu) in the diet is catabolized by the small intestine mucosa [2,17]. Infections and some types of long-term exercise have also been shown to increase Gln consumption, thereby reducing the levels of this amino acid in blood and other tissues [15,19]. Prolonged exercise or periods of strenuous exercise have been shown to lower Gln levels, and it has been suggested that this reduction may be one of the causes of exercise-induced immune suppression syndrome [3].

Given the characteristics of glutamine metabolism and the difficulty of evaluating the effects of dietary protein intake on blood Gln levels, an experiment was devised to supplement horses with different inclusions of a combination of glutamine and glutamate in the concentrate in order to characterize the rate of glutamine utilization in the blood of healthy horses. This information may help shed light on supplementation with these amino acids for horses subjected to different training loads.

MATERIALS AND METHODS

Animals

This study involved four healthy adult (~13 years old) purebred Arabian mares, not in training, with a body mass of 380 kg, all vaccinated and dewormed regularly which were housed at the Center for Equine Research, Department of Animal Science, Federal Rural University of Pernambuco - UFRPE. The procedures used were approved by the institution's animal ethics and welfare committee (Number: 62/2007-CTA/DZ- UFRPE).

Supplementation and experimental model

The animals were fed as recommended by the National Research Council [12] for horses not in training.

Fifty percent of their energy requirements were supplied by concentrate Equimax Premium^{®1}, which was divided into two meals of the same amount (at 6 am and 5 pm). The other 50% came from Tifton hay (*Cynodon dactylon*), which was supplied *ad libitum*. The Animals had free access to water and mineral salt. In the experimental model, the mares were distributed in a Latin square design comprised of four treatments: control (without inclusion) and inclusions of 1%, 2% and 4% of supplement AminoGut^{®2}, and four animals. During each 7-day phase, the animals were fed the supplement mixed with commercial concentrate once a day in the morning. AminoGut[®] contains a combination of 10% glutamine and 10% glutamate. A 7-day washout period was established between each phase. A sample of the concentrate was analyzed at the Animal Nutrition Laboratory of the Federal University of Ceará, while the nonstructural carbohydrates and the area under the curve were calculated according to the model described by literature [6].

Blood collection and laboratory analysis

Fasting blood samples (T0) were drawn after a 12-hour fast, followed by samples taken at 60 min (T60), 120 min (T120), 240 min (T240) and 360 min (T360)) after supplementation with the concentrate in the different treatments. The blood was drawn into pre-cooled heparinized vacuum tubes, after which a 0.5 mL aliquot of plasma was deproteinized in 0.5 mL of 10% perchloric acid (PCA) solution and then centrifuged. The resulting supernatant was neutralized with sodium hydroxide (KOH) and stored at -20°C for later determination of glutamine (Gln) and glutamate (Glu) concentrations. Glutamine (Gln) and Glutamate ([Glu]) concentrations were analyzed as described in the literature, using the enzymatic spectrophotometric method [11].

The remainder of the blood was then divided into two new aliquots, one of which was used to determine hematocrit levels by the micro hematocrit concentration technique, while the other was centrifuged to obtain plasma. This was used to measure Total Plasma Protein (TPP) by refractometry and Glucose by the tape test Accu-Chek^{®3}. Urea (URE), Creatinine (CREAT) and Uric Acid (UrAc) were measured using commercial kits Doles Reagents^{®4} in a semi-automated analyzer Doles D2504.

Statistical analysis

The results of the laboratory analyses were subjected to one and two-way ANOVA (time and treatment), and to Tukey's method as a post hoc test,

using SigmaPlot version 13 software for Windows. *P* was set at 5% in all the cases. Results are expressed as mean +/- mean standard error.

RESULTS

The findings indicate that Gln changed as a function of both treatments and blood collection time ($P < 0.001$), but no interaction occurred between these two variables [time and treatment] ($P > 0.05$) [Table 1]. An analysis of the different treatment phases indicated that Gln changed significantly at T360 of the 4% treatment when compared with the fasting phase of all treatments, and at T60 and T240 of the control treatment ($P < 0.05$). All the other biomarkers analyzed here were unchanged [Glutamate, Urea, Creatinine, Urea, TPP, Glucose and Hematocrit] ($P > 0.05$) during the period under analysis, and remained within the normal range for the species in their current stabling conditions (Tables 1, 2 and 3). The mares presented no clinical problems nor did they change their feeding behavior during the supplementation period or on the days blood was collected. The analysis of the concentrate used in all the experiments indicated that it contained 18.87% crude protein (CP), 7.95% ether extract, and 4.1 Mcal of gross energy from dry matter, while its nonstructural carbohydrate content was estimated at 38.36%. An analysis of the area under the Gln curve of the different treatments revealed a tendency for differences between the treatments ($P = 0.06$), and the control treatment presented the smallest area (~2,000), followed by the treatment involving the inclusion of 2% Gln+Glu (~2,674). The treatments with of 1% and 4% inclusions presented larger and similar areas under the curve (~2,800).

DISCUSSION

Glutamine metabolism in horses has yet to be extensively studied. However, a previous study [7] showed that, when supplied to horses in its free form, this amino acid causes Gln levels to rise rapidly within the first 90 min of the postprandial period. This indicates that an extra amount of this amino acid may increase Gln blood levels despite intense degradation of enterocytes [2,17]. This study found that supplementation with a combination of Gln+Glu can increase blood Gln levels after 6 h in the treatment involving 4% Gln+Glu included in the concentrate ($P < 0.05$). Moreover, this increase was found to be significant

only after 6 h, but represented an increase of almost 50% when compared to the control levels in the same phase. Differences in the peak blood Gln levels in the two experiments described here may indicate that the different formulations that were used to supply glutamine may allow for different therapeutic applications of this amino acid. Another important point is that, given this variation in the postprandial peak of glutamine levels, this amino acid can be used to obtain the maximum supplementation at the desired time and should be carefully evaluated in different products to optimize its use.

Gln supplementation may be important to increase the blood levels of this amino acid, since it has been reported that Gln blood levels decreased after exercise in different sport modalities. An earlier study [15] showed that equestrian horses could have lower blood Gln when simulating the cross-country phase of a full riding competition. Moreover, some time later, other study [19] demonstrated that blood Gln levels of Mangalarga Marchador horses are decreased (~25%) 4 h after the completion of a standard gait test. It has also been reported that Gln and Glu levels in Standardbred horses are significantly lowered after an intense training program [20] and after short bursts of intense exercise [5]. Some of these authors did not find changes in blood Glu levels in their experiments [15,19,20]. Therefore, determining how different supplements can raise Gln levels may contribute to the elevation or maintenance of the levels of this amino acid in the blood, thus preventing possibly reduced availability for the cells of the intestines and immune system.

Given the difficulties of Gln supplementation and the fact that enterocytes are important consumers of this amino acid, different authors have been looking for ways to increase the blood levels of this amino acid by improving fat-free mass through supplementation with combinations of amino acids and nutraceuticals. Based on the idea of combined nutraceutical and amino acid supplementation, it has been shown that this formulation strategy increases Gln levels after 12 weeks of supplementation in working horses [8], but the same combination does not produce similar effects when used on elderly horses more than 20 years old [9]. However, it should be noted that the glutamine metabolism of older horses is impaired by senility, because even elderly horses with high Gln levels in their muscle tissue do not necessarily have high levels in their plasma, as in adult animals [10].

Table 1. Blood levels of glutamine and glutamate in horses not in training after the inclusion of a mixture of glutamine and glutamate in the concentrate, determined by two-way ANOVA (Treatment vs. Phase).

Biomarker	Phase					P value		
	Fasting	60 min	120 min	240 min	360 min	Treat	Phase	Interaction
Glutamine, $\mu\text{mol/mL}$								
Treat Control	0.307 \pm 0.051 [#]	0.303 \pm 0.051 [#]	0.385 \pm 0.051	0.312 \pm 0.051 [#]	0.359 \pm 0.051	< 0.001	< 0.001	> 0.05
Treat 1%	0.332 \pm 0.51 [#]	0.432 \pm 0.051	0.400 \pm 0.051	0.535 \pm 0.051	0.438 \pm 0.051			
Treat 2%	0.327 \pm 0.051 [#]	0.437 \pm 0.051	0.466 \pm 0.051	0.461 \pm 0.051	0.559 \pm 0.059			
Treat 4%	0.303 \pm 0.051 [#]	0.524 \pm 0.051	0.558 \pm 0.051	0.497 \pm 0.051	0.602 \pm 0.051 [#]			
Glutamate, $\mu\text{mol/mL}$								
Treat Control	0.042 \pm 0.014	0.082 \pm 0.014	0.058 \pm 0.014	0.073 \pm 0.014	0.060 \pm 0.014	> 0.05	> 0.05	> 0.05
Treat 1%	0.056 \pm 0.014	0.074 \pm 0.014	0.066 \pm 0.014	0.059 \pm 0.014	0.049 \pm 0.014			
Treat 2%	0.050 \pm 0.016	0.069 \pm 0.014	0.086 \pm 0.014	0.067 \pm 0.014	0.079 \pm 0.016			
Treat 4%	0.050 \pm 0.014	0.056 \pm 0.014	0.072 \pm 0.014	0.082 \pm 0.014	0.098 \pm 0.014			

[#] indicates that $P < 0.05$ by Tukey's test, regardless of line or column for the same biomarker, as compared to the 360 min phase of 4% treatment; min= minutes; Treat= treatment.

Table 2. Blood levels of urea, creatinine and uric acid in horses not in training after the inclusion of a mixture of glutamine and glutamate in the concentrate, determined by two-way ANOVA (Treatment vs. Phase).

Biomarker	Phase					P value		
	Fasting	60 min	120 min	240 min	360 min	Treat	Phase	Interaction
Urea, $\mu\text{mol/mL}$								
Treat Control	8.79 \pm 1.09	8.84 \pm 1.09	8.82 \pm 1.09	8.97 \pm 1.09	9.20 \pm 1.09	> 0.05	> 0.05	> 0.05
Treat 1%	8.22 \pm 1.09	8.46 \pm 1.09	8.35 \pm 1.09	7.76 \pm 1.09	8.51 \pm 1.09			
Treat 2%	8.18 \pm 1.09	8.42 \pm 1.09	8.43 \pm 1.09	8.55 \pm 1.09	8.60 \pm 1.09			
Treat 4%	8.17 \pm 1.09	8.47 \pm 1.09	8.19 \pm 1.09	8.61 \pm 1.09	8.86 \pm 1.09			
Creatine, $\mu\text{mol/mL}$								
Treat Control	0.12 \pm 0.01	0.12 \pm 0.01	0.12 \pm 0.01	0.13 \pm 0.01	0.12 \pm 0.01	> 0.05	> 0.05	> 0.05
Treat 1%	0.12 \pm 0.01	0.12 \pm 0.01	0.12 \pm 0.01	0.12 \pm 0.01	0.11 \pm 0.01			
Treat 2%	0.12 \pm 0.01	0.13 \pm 0.01	0.12 \pm 0.01	0.12 \pm 0.01	0.12 \pm 0.01			
Treat 4%	0.13 \pm 0.01	0.13 \pm 0.01	0.12 \pm 0.01	0.12 \pm 0.01	0.12 \pm 0.01			
Uric Acid, mmol/L								
Treat Control	0.03 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01	> 0.05	> 0.05	> 0.05
Treat 1%	0.03 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01			
Treat 2%	0.03 \pm 0.01	0.04 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01			
Treat 4%	0.03 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01			

min= minutes; Treat= treatment.

These conflicting results indicate that there are particularities in the use of different supplements and their effect on the variation of different biomarkers in animal blood, particularly of amino acids, and these particularities may include the animal's age and the supplement formulation.

Decreased Gln levels in human athletes are attributed to overtraining or occur after long-term exercise, and this may be associated with the onset of

disorders in the recovery phase of competitions, since white blood cells use this amino acid as an energy source [1,14]. The decrease in Gln may reduce the availability of these amino acids for consumer tissues, such as enterocytes and cells of the immune system, favoring the onset of disorders or aggravating existing clinical conditions [18]. This process may be severe in older animals, because of their reduced ability to absorb

Table 3. Blood levels of TPP, hematocrit and glucose in horses not in training after the inclusion of a mixture of glutamine and glutamate in the concentrate, determined by two-way ANOVA (Treatment vs. Phase).

Biomarker	Phase					P value		
	Fasting	60 min	120 min	240 min	360 min	Treat	Phase	Interaction
TPP, g/L								
Treat Control	62 ± 1.5	62 ± 1.5	61 ± 1.5	60 ± 1.5	60 ± 1.5	> 0.05	> 0.05	> 0.05
Treat 1%	62 ± 1.5	61 ± 1.5	60 ± 1.5	60 ± 1.5	59 ± 1.5			
Treat 2%	63 ± 1.5	65 ± 1.5	60 ± 1.5	62 ± 1.5	62 ± 1.5			
Treat 4%	62 ± 1.5	63 ± 1.5	62 ± 1.5	62 ± 1.5	63 ± 1.5			
Hematocrit %								
Treat Control	27.75 ± 1.20	26.50 ± 1.20	25.75 ± 1.20	25.50 ± 1.20	25.50 ± 1.20	> 0.05	> 0.05	> 0.05
Treat 1%	28.67 ± 1.39	24.50 ± 1.20	24.75 ± 1.20	24.75 ± 1.20	25.25 ± 1.20			
Treat 2%	27.33 ± 1.39	27.00 ± 1.20	26.25 ± 1.20	24.50 ± 1.20	26.50 ± 1.20			
Treat 4%	27.00 ± 1.39	25.75 ± 1.20	25.25 ± 1.20	26.00 ± 1.20	27.25 ± 1.20			
Glucose, mmol/L								
Treat Control	5.61 ± 0.99	5.33 ± 0.99	4.62 ± 0.99	5.66 ± 0.99	3.95 ± 0.99	> 0.05	> 0.05	> 0.05
Treat 1%	4.52 ± 0.99	5.06 ± 0.99	5.39 ± 0.99	4.61 ± 0.99	4.11 ± 0.99			
Treat 2%	3.87 ± 0.99	5.41 ± 0.99	5.26 ± 0.99	4.61 ± 0.99	4.46 ± 0.99			
Treat 4%	5.39 ± 0.99	6.72 ± 0.99	6.33 ± 0.99	5.19 ± 0.99	4.24 ± 0.99			

min= minutes; Treat= treatment.

food nutrients and the low immune response typical of senility [10]. Therefore, dietary supplementation with this amino acid could be used to raise or maintain its blood levels in horses, counteracting the negative effects of intense training or hastening recovery after competitions, or even preventing respiratory disorders. No study of supplementation has been conducted with sick animals, but it has been shown that horses infected with equine influenza virus show significantly lower Gln levels [15]; hence, supplementation with Gln might reverse this process and hasten recovery.

In the postprandial period, Gln levels do not change in healthy adult horses [16], unlike what has been observed in young animals [4], but these levels may vary in horses, depending on their development status and lactation. Also, it was demonstrated that, during lactation, the Gln blood levels of mares remain high in the first seven days after delivery, but decline significantly at the end of lactation [9]. Changes in Gln levels in foals from birth to 12 months of age have also been demonstrated, with Gln levels rising (~50%) in the days following birth, as well as glutamine synthetase expression [11]. These variations associated with the development or reproductive stage of females can also influence the behavior of glutaminemia in horses, and should also be taken into account when setting up a supplementation program for these groups.

It has also been reported that supplementation with L-Glutamine does not change postprandial ammonia concentrations [7]. Mean urea, creatinine, uric acid, total plasma protein, glucose and hematocrit levels were within the normal range for horses [13] and did not vary significantly throughout the postprandial period ($P > 0.05$) [Tables 2 and 3]. The absence of variations in total plasma protein and hematocrit levels indicate that the mares did not undergo significant variations in plasma volume during the blood collection phase, which would have compromised some of the results found in this experiment. A greater variation in glucose levels was expected, but the amount of concentrate supplied may not have been sufficient to produce changes in the levels of this biomarker. Moreover, the concentrate used here did not contain high non-structural carbohydrate content, which was lower than 40%. Lastly, it should be noted that hematocrit levels remained unchanged, indicating that the animals remained calm and did not undergo any kind of stress that could stimulate splenic contraction, thereby modifying the percentage of this hematological parameter.

CONCLUSIONS

It was concluded that supplementation with a combination of glutamine plus Glutamate may increase blood glutamine levels in healthy horses. Such

supplementation may therefore be used preventively or as a treatment in cases when the level of this amino acid in horses is expected to be low, in situations where the catabolism of this amino acid is high, such as during lactation and after low intensity and long duration exercise. Treatment with the inclusion of 4% GLN+GLU in the feed not only produced the highest increases in glutamine levels but also had the strongest postprandial effect, and can therefore be recommended as the best percentage of dietary inclusion for horses in these conditions.

MANUFACTURERS

¹IRCA Animal Nutrition. Carpina, PE, Brazil.

²Ajinomoto do Brasil. São Paulo, SP, Brazil.

³Roche Diagnostics. São Paulo, SP, Brazil.

⁴Doles Reagentes e Equipamentos para Laboratório. Goiânia, GO, Brazil.

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Ethical approval. All the procedures employed in this study were approved by the institution's animal ethics and welfare committee (# 62/2007-CTA/DZ- UFRPE, Federal Rural University of Pernambuco).

Declaration of interest. The authors declare that there are no conflicts of interest.

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