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Erythrocyte vitamin B_2 and B_6 dependent enzymes in adult and newborn Thoroughbred horses

Enzimas eritrocitárias dependentes das vitaminas B_2 e B_6 em cavalos recém-nascidos e adultos Puro-Sangue Inglês

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SUMMARY

In order to assess the Thoroughbred horses nutritional status as to riboflavin and pyridoxine, 10 adults and 30 newborns were investigated. In both adults and newborns, riboflavin intake was sufficient to meet their nutritional needs; however, a moderate pyridoxine deficiency was detected amongst the adult horses, but not in the newborns. This findings suggests that a pyridoxine supplementation to adult horses may be necessary to keep their nutritional poise.

UNITERMS: Thoroughbred horses; Erythrocytes; Riboflavin; Pyridoxine; Glutathione reductase; Aspartato-aminotransferase.

INTRODUCTION

Keeping up the nutritional state of different animal species has been a challenge to zootechnicians and veterinarians who work in farms and zoos or take care of pet animals. Among the usual nutritional parameters, the water soluble complex B vitamins play an important role as these vitamins are involved in many crucial metabolic pathways. The vitamin B_2 5' - phosphoric ester, the flavin mononucleotide (FMN) -, is an important oxidation-reduction coenzyme participating in cell respiration, besides being a precursor in the synthesis of flavin adenine dinucleotide (FAD) - another important oxidation-reduction coenzyme. Both FMN and FAD act as prosthetic group for flavin dehydrogenases. The phosphate ester of vitamin B_6, the pyridoxal-phosphate, is an essential growth factor for animal cells, acting as a prosthetic group of transaminases and other enzymes involved in the alpha-aminoacid decarboxilation, in the removal of sulphur from cysteine and dehydration of serine.

In order to assess the nutritional state of adult and newborn Thoroughbred horses in relation to B_2 and B_6 vitamins, we employed an indirect method based upon the assays of red cell glutathione reductase (GR) and glutamate oxaloacetic transaminase (GOT) activities (Beutler[5], 1984).

Abbreviations:

ACD: citric acid, citrate, dextrose; EDTA: ethylenediaminetetraacetate; HPLC: high performance liquid chromatography; NADH: reduced nicotinamide adenine dinucleotide; NADPH: reduced nicotinamide adenine dinucleotide phosphate; RBC: red blood cells; Tris: tris(hydroxymethyl) nitromethane; GR: glutathione reductase; GOT: glutamate oxaloacetate transaminase; FAD: flavin adenine dinucleotide; FMN: flavin adenine mononucleotide; PyrP: pyridoxal phosphate; GR-AC: GR Activity Coefficient ratio with FAD / without FAD; GOT-AC: GOT Activity Coefficient ratio with PyrP / without PyrP

As glutathione reductase has a FAD containing prosthetic group, its activity is dependent on the availability of riboflavin (Beutler[4], 1969; Bamji[1], 1969; Turham[13], 1981; Bates *et al.*[3]; 1982); so, if FAD is added *in vitro* to the reagent system, the increase of its activity will be directly proportional to the degree of riboflavin deficiency. An activity coeficient has been used to assess the riboflavin blood levels (Coopermann *et al.*[7], 1973). An index greater than 1.2 in glutathione reductase with FAD/glutathione reductase without FAD ratio is indicative of riboflavin deficiency. However, using Beutler's[5] data (1984) of glutathione reductase activities with/without FAD for a normal human population, this index could increase to 1.4, and any value above this level would indicate riboflavin deficiency.

In a similar way, the vitamin B_6 deficiency may be accurately detected by assaying the glutamate oxaloacetic transaminase activity (GOT). It is known that GOT contains pyridoxal phosphate as prosthetic group. The *in vitro* increase of activity resulting from pyridoxal phosphate addition system is directly proportional to the vitamin B_6 deficiency, and an index greater than 1.69 in GOT with pyridoxal/GOT without pyridoxal ratio unveils a vitamin B_6

deficiency (Salked *et al.*[12], 1973).

Although the Thoroughbred horses are usually fed upon fine and balanced diet they are special athletes with special needs, we carried out this study to detect any deficiency regarding vitamin B complex, especifically riboflavin and pyridoxine.

MATERIAL AND METHODS

Blood samples from 10 adult Thoroughbred horses, as well as from 30 newborns at delivery and at 3 and 7 days after birth were collected in ACD and kept at 4°C up to 4 days for processing. The red blood cells were washed three times for 10 minutes in saline at 4°C at 3000 x g, and one volume of packed red cells was lysed in 20 volumes of hemolysing solution containing 2.7 mM EDTA, 0.005 mM betamercaptoethanol. Freeze-and-thawing procedure was employed, the suspension was centrifuged at 5000 x g, and the supernatant was used for enzymes assays, in a Gilford 2400 recorder spectrophotometer, at 37°C, at 340 nm. Sigma products were used when not indicated.

The first reagent system for glutathione reductase assay without FAD (Beutler[5], 1984) contained 50 mM Tris-HCL, 0.25 mM EDTA pH 8.0, 3.3 mM GSSG, 0.1 mM NADPH, 0.010 ml of 1:20 hemolysate (total volume of 1 ml). The second reagent system was the same except for the addition of 0.001 mM FAD, and the activity coefficient was obtained by the ratio: activity with FAD/activity without FAD (Beutler[5], 1984).

The first reagent system employed for glutamate oxaloacetate transaminase assay was a Boehringer Mannheim system which contained the following reagents: 80 mM phosphate buffer pH 7.4, 0.020 ml of 1:20 hemolysate, 200 mM L-aspartate, 0.6 I.U. malate dehydrogenase, 1.2 I.U. lactate dehydrogenase, 0.18 mM NADH, 12 mM alphaketoglutarate. The second system was the same except for the addition of 0.020 mM pyridoxal phosphate, and the activity coefficient was obtained by the ratio: activity with PyrP/activity without PyrP (Beutler[5], 1984).

The activities were calculated as I.U. per gram of hemoglobin per minute at 37°C (Beutler[5], 1984). Hemoglobin estimation was performed by cyanmethaemoglobin conversion, at 540 nm.

RESULTS

The results are depicted in Tab. 1 and 2.

DISCUSSION

Glutathione reductase. The glucose metabolism through the pentose pathway comprises 13% of the total utilized glucose in the erythrocyte of the Thoroughbred horses (Harvey; Kaneko[9], 1976), a value much higher than the 10% observed in human red cells. This fact may hint the importance of NADPH generation by the pentose shunt for the horse red cells.

According to Robin; Harley[11] (1967), there is greater erythrocyte reduced glutathione depletion in horses than in human blood. This suggests a clamant need for reducing power in horse erythrocytes, when compared to humans. According to our data (Table 1), there is a greater glutathione reductase activity in newborns than in adults, and the 30% increase in its activity by FAD addition discloses an adequate riboflavin availability for newborn and adult horses as well, although the GR-AC in the newborn RBC showed slightly lower than the GR-AC found in adult horses. As in newborn horses there are 3 clearly defined periods soon after delivery, v.g. umbilical cord blood (at delivery), the hemolytic period (up to the 3rd day) and the post-hemolytic period (up to the 7th day) (Medeiros *et al.*[10], 1982), we have also studied the glutathione reductase in these periods, and did not find any significant variation

Table 1

Erythrocyte glutathione reductase in adult and newborn Thoroughbred horses*. System #1 (FAD): 50 mM Tris-HCl pH 8.0, 25 mM EDTA, 33 mM GSSG, 0.1 mM NADPH, 0.010 ml of 1:20 helolysate; 0.001 Mm FAD. System #2 (no FAD): 50 mM Tris-HCl, ph 8.0; 25 mM EDTA; 33 mM GSSG; 0.1 mM NADPH; 0.01 ml of 1:20 hemolysate. São Paulo, 1991.

System	Adults (n:10)	Newborns (n:30)		
		Umbilical cord blood	3 days-old	7 days-old
1 (FAD)	4.3 ± 0.4	5.8 ± 0.6	5.8 ± 0.6	5.7 ± 0.5
2 (no FAD)	3.2 ± 0.2	4.5 ± 0.5	4.7 ± 0.5	4.6 ± 0.7
GR-AC (1/2)	1.34 ± 0.1	1.29 ± 0.1	1.24 ± 0.1	1.24 ± 0.1

* Enzyme units in I.U. g Hb^{-1} .min^{-1} at 37°C.

along them, as shown in Tab. 1. The absence of variation along the 3rd and 7th day after birth suggests good riboflavin intake supplied by maternal milk. The glutathione reductase activity in the newborns is about 30% greater than in adults, which may be explained by the presence of a greater percentage of young red cells or by a foetal pattern in the newborn red cells. As both adult and newborn horses presented the same activity coefficient for red blood cell glutathione reductase activity assayed in the presence or in the absence of coenzyme, we may, therefore, suggest an equal availability and utilization of riboflavin.

Glutamate oxaloacetate transaminase. Regarding the red cell glutamate oxaloacetate transaminase, there is an unexpected higher activity in adults than in newborns, in contrast to what happens to humans (Glendening *et al.*[8], 1955; Bamji[2], 1976). This behaviour suggests a foetal pattern, which will be replaced by an adult pattern at later stages of development. The foetal pattern, however, is kept throughout the first week after birth, although great changes do occur in this period, with deep hemolysis and exceeding percentage of new circulating red cells (Medeiros *et al.*[10], 1982). Therefore, definitive changes must occur later.

Although the adult horses present a high GOT activity, its *in vitro* 71% stimulation (Tab. 2) by pyridoxal phosphate suggests there is a significant percentage of coenzyme non-saturated apoenzyme, in spite of well balanced diet given to these animals. Inasmuch as it is accepted that AC greater than 1.7 indicates vitamin B_6 deficiency in humans, this borderline value obtained from adult horses suggests that these horses would benefit from a supplementary vitamin B_6, mostly when the importance of GOT in the interconversion of metabolic pathways is known, besides the pyridoxal phosphate role in the globin synthesis.

Although it may be claimed that horses have little to do with humans, as to the processes herein investigated, and that an AC of 1.7 for humans may not be held for horses, it should be noted that the GOT-AC for newborn RBC is 1.4, or 30% smaller than adults. Certainly, the best figures observed in infant horses in this series suggest that adult horses would take advantage from such a pyridoxine supplementation as they seem to present a moderate pyridoxine deficiency, probably due to greater needs. Coburn *et al.*[6] (1984), employing HPLC, apotyrosine decarboxilase activity and radiometric methods observed a wide range in vitamin B_6 concentrations between species, and suggested that there may be differences in dietary intake and or metabolism of vitamin B_6.

Table 2

Erythrocyte glutamate oxaloacetic transaminase in adult and newborn Thoroughbred horses*. System #1 (Pyr-P): 80 mM phosphate buffer pH 7.4, 200 mM L-aspartate, 0.6 I.U. malate dehydrogenase, 1.2 I.U. lactate dehydrogenase, 0.18 mM NADH, 12 mM alpha-ketoglutarate, 0.02 ml of 1:20 hemolysate; 0.02 mM PyrP. System #2 (no Pyr-P): System #2 (Pyr-P): 80 mM phosphate buffer pH 7.4, 200 mM L-aspartate, 0.6 I.U. malate dehydrogenase, 1.2 I.U. lactate dehydrogenase, 0.18 mM NADH, 12 mM alpha-ketoglutarate, 0.02 ml of 1:20 hemolysate. São Paulo, 1991.

System	Adults (n:10)	Newborns (n:30)		
		Umbilical cord blood	3 days-old	7 days-old
1 (PyrP)	3.6 ± 0.9	2.1 ± 0.5	1.8 ± 0.6	2.1 ± 0.5
2 (no PyrP)	2.1 ± 0.55	1.5 ± 0.5	1.3 ± 0.6	1.5 ± 0.4
GOT-AC (1/2)	1.71 ± 0.2	1.4 ± 0.13	1.38 ± 0.15	1.4 ± 0.13

* Enzyme activities in I.U. g^{-1} Hb. min^{-1} at 37°C.

RESUMO

Com o propósito de determinar o estado nutricional de cavalos Puro-Sangue Inglês (PSI) em relação à riboflavina (vitamina B_2) e à piridoxina (vitamina B_6), 10 animais adultos e 30 recém-nascidos foram investigados. Foi observado um bom estado nutricional quanto à riboflavina, notando-se moderada deficiência de piridoxina nos animais adultos, mas não nos recém-nascidos. Estes fatos sugerem que os animais adultos devam receber suplementação com piridoxina.

UNITERMOS: Cavalos Puro-Sangue; Eritrócitos; Riboflavina; Piridoxina; Glutationa redutase; Aspartate-aminotransferase.

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