The Influence of Exercise on the Daily Rhythm of Serum Homocysteine in Horses

F. FAZIO1, A. ASSENZA1, G. CRISAFULLI2, G. PICCIONE1, and G. CAOLA1

1Department of Morphology, Biochemistry, Physiology and Animal Production. Veterinary Chronophysiology Laboratory. Section of Veterinary Physiology, Faculty of Veterinary Medicine, University of Messina, Polo Universitario dell’Annunziata, 98168 Messina, Italy; and 2U.O.C. of chemical biochemistry, A.O.U. “G. Martino”, 98100 Messina, Italy

Abstract: The aim of this study was to determine the daily rhythms in the blood serum of homocysteine in horses. Ten thoroughbred horses, five athletic (trained for 1 h, 6 days a week) and five sedentary, were used. Blood samples were collected on each subject every 4 h for two days by means of the jugular vein. On each individual sample, the serum concentration of homocysteine was assessed. The results obtained during the experimental period indicated the existence of a daily rhythm of serum homocysteine in sedentary and athletic horses. They also demonstrated that in horses, physical exercise influences the daily rhythm of serum homocysteine.

Key words: circadian rhythms, homocysteine, horse.

Homocysteine (Hcy) is a sulfur intermediate amino acid in methionine-cysteine metabolism [1]. The importance of this metabolic pathway relates to the sheer number of physiological functions in which it is involved; it contains the methyl donor (S-adenosyl-methionine) for virtually all methylation reactions in the organism [2] and provides the limiting substrate (cysteine) for the synthesis of glutathione [3]. The metabolism of Hcy leads to three possible main outcomes: it may be remethylated to methionine, converted to cysteine by transulfuration, or exported to the extracellular space. In man, moderately increased concentrations of total homocysteine (tHcy) have been identified as an independent risk factor for cardiovascular disease (CVD), stroke, and other thrombotic events [4–11], as well as increased concentrations of total cysteine (tCys) [12]. Regular physical exercise is now recognized to reduce the risk of CVD development [13, 14]. Sedentary subjects, however, are considered to be at risk for CVD. Although available evidence indicates that physical activity modifies the lipid and carbohydrate metabolism [15], non energetic exercise-induced metabolism stress has not been extensively studied [16]. Recent data have pointed out that the duration and intensity of exercise are relevant factors in modulating the plasma Hcy levels. Bailey et al. [17] have shown that a 4-week exercise program was associated with a reduction in Hcy levels in healthy men, whereas Nygard et al. [18] confirmed that a lack of exercise might be partly associated with elevated plasma tHcy levels, increasing the risk of CVD in the elderly. As far as horses are concerned, there are not many studies on homocysteine, neither on the effects derived from training nor on its daily rhythm. The few that are available deal with the physiological range of Hcy and its variations in relation to some pathologies [19] and the influence of a single exercise test, but not training, on the plasmatic levels of homocysteine [20]. As for studies on daily rhythm in men [21–23] and rats [24, 25], it is evident that the rhythmicity of the parameter and the influence of physical exercise have been considered only together, never separately. It is known that many circadian rhythms in physiological functions are evident under resting conditions and that these rhythms are influenced by physical exercise [26]. In regard to the athletic horse, chronobiological studies have detected several physiological parameters that feature a distinctive circadian rhythmicity; they include body temperature, hematologic condition, cardiovascular indexes, and the serum-branched chain amino acids tyrosine, tryptofane, and serotonin [27–29]. The existence of circadian rhythms in exercise performance and in response to exercise merits detailed exploration. To conclude, considering the lack of data in the present literature and following our studies on the daily rhythm applied to athletic horses, we aimed to determine if training may also influence a possible circadian rhythm alongside an effect on the levels of serum homocysteine. This seems to be particularly interesting in view of a more appropriate interpretation of the physiological effects of physical exercise on the daily rhythm of serum homocysteine.
Materials and methods

Ten thoroughbred horses were used (females, 7–9 years old, 530 ± 20 kg of body), five athletic and five sedentary. They were subjected to the same type of management: individual boxes, natural winter photoperiod (sunrise at 07:00, sunset at 17:30), natural indoor temperature (17–19°C), and the same feeding schedule of hay and oats twice a day (08:00 and 18:00). Water was available ad libitum. The five designated athletic underwent fitness training for 60 days prior to the experimental test, 6 days a week with a rest day on Sundays. Training lasted 1 h (16:00) every day and included walking, trotting, galloping, and obstacle jumping. Sedentary horses were always kept inside their stalls with no physical activity. Blood samples were collected at 4-h intervals over a 48-h period (starting at 08:00 on day 1 and finishing at 04:00 on day 2) via an intravenous cannula inserted into the jugular vein. Blood samples have been centrifuged at 2,000 × g for 20 min. Serum was stored at −20°C until analyzed. Serum total homocysteine values were determined by high performance liquid chromatography (HPLC – Agilent 1100, BIO-RAD) with fluorimetric detection and isocratic elution [30]. This methodology involves three steps, namely, a reduction of thiol groups using TCEP [tris(CarboxyEthyl)Phosphine], protein precipitation, and derivatization with SBD-F (7-fluorobenzene-2-oxy-1,3-diazolic-4-ammonium sulfate). The HPLC system used was a Shimadzu apparatus with an SIL-10ADvp automatic sample injector and an RF-10AXL fluorescence detector. Chromatographic separation was performed using a C18 model Shim-pack CLC-ODS column (4.6 × 150 nm with 5.0 µm microparticles). The fluorescence of the separated compounds was measured with a detector adjusted for excitation at 385 nm and emission at 515 nm. The total Hcy concentrations were calculated with a calibration curve by using known amino acid concentrations and cystamine as the internal standard. All the results were expressed as mean ± SD. A one-way repeated measures analysis of variance (ANOVA) was used to determine significant differences. P values <0.05 were considered statistically significant. The data were analyzed by using the software STATISTICA 5.5 (StatSoft Inc., USA). The acrophase of a rhythm was determined by an iterative curve-fitting procedure based on the single cosinor procedure [31]. All work presented here complies with current regulations covering animal experimentation in Italy.

Results

The results obtained during the experimental period indicate the existence of a daily rhythm of serum homocysteine in the sedentary and athletic horses, as shown in the figure. The ANOVA showed a highly significant effect of time in all sedentary and athletic horses (sedentary: $F_{(11,44)} = 2.37, p < 0.02$; athletic: $F_{(11,44)} = 3.92, p < 0.05$).
0.0005). The application of the periodic model and a statistical analysis of the cosinor enable us to define the periodic parameters and their acrophases (expressed in hours) during the 2 days of monitoring. In sedentary horses, serum Hcy showed diurnal acrophases at 09:40 (day 1) and at 10:24 (day 2), but in athletic horses Hcy showed nocturnal acrophases at 22:12 (day 1) and at 22:04 (day 2). The table shows the MESOR, with the fiducial limits (F.L.) at 95%; the amplitude, expressed in the same unit as the relative MESOR; the acrophase, calculated using the singular cosinor method and expressed in hours, together with the confidence interval at 95%, for the periodic serum homocysteine in the sedentary and athletic horses.

**Discussion**

In our study, the serum Hcy value was within the physiological range for horses [19]. The results obtained in the study outline a daily rhythm of serum Hcy in the sedentary and athletic horses. In sedentary horses, acrophases occurred during the diurnal hours at 09:40 (day 1) and at 10:24 (day 2); in athletic horses, they occurred during the nocturnal hours at 22:12 (day 1) and at 22:04 (day 2). Daily rhythm in plasma Hcy may reflect variation in the activity of the cellular export mechanism, which will result in varying levels of Hcy being dispersed to the plasma at different phases of the 24 h rather than in its rate of metabolism [21]. In a study related to the circadian rhythm of Hcy in humans [22], the highest levels were reported at around 22:00, gradually decreasing thereafter to the lowest levels at around 10:00. Other research reported the highest Hcy levels in rats at night around 02:00, which are different from those in humans [24]. These results show that the homocysteine rhythm is different in rats and humans. It is postulated that these differences possibly derive from different activity patterns and feeding times. Although Hcy rhythm has been studied in detail in men and rats, at present there are no studies where a relation has been made between the variations of serum Hcy as the effect of physical exercise and the influence of physical exercise on the circadian rhythm. Nevertheless, the effects of exercise, training in particular, on several physiological-metabolic parameters in men [26] and horses are well known. We showed, for example, in our previous research the existence of the circadian rhythm of tryptophan and serotonin in sedentary and athletic horses. It is interesting that both parameters changed their acrophases in athletic horses trained in the afternoon [28]. Consequently, because the sedentary horses have been maintained under the same conditions of housing, illumination, and feeding schedule as the athletic horses, it can be assumed that the afternoon physical exercise of the athletic horses might determine the change in the acrophase of serum Hcy rhythm. Although we consider the findings of the present research to be preliminary, it cannot be denied that they might be used for a much clearer interpretation of physiological reference values of serum Hcy in horses and for a more correct interpretation of their variations during acute and chronic exercise. For this reason, we consider the continuance of our studies of great importance to further explain the role of physical exercise as the Zeitgeber of serum homocysteine circadian rhythms.

**REFERENCES**


