ORIGINAL ARTICLE

The role of the light/dark cycle in the daily rhythm of serum proteins in *Equus caballus*

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Summary

This research was carried out on five clinically healthy Sella Italiana horses to determine the daily rhythm of total proteins and their fractions, to establish if these rhythms are endogenously generated and to assess the role of light as synchroniser of these rhythms. Blood samples were collected from each subject every 3 h over a period of 48 h, starting at 9:00 on day 1 and finishing at 9:00 on day 3, into vacutainer tubes without an anticoagulant via intravenous cannulas inserted into the jugular vein. Total serum proteins, albumin, α_1 -, α_2 -, β_1 -, β_2 - and γ -globulin concentrations were assessed in all samples. The application of two-way ANOVA showed a significant effect of the time of day on total proteins, albumin, β_1 - and β_2 -globulins, and of the experimental conditions on total proteins α_1 -, α_2 -, β_1 -, β_2 - and γ -globulins. No statistical modifications were observed on the A/G ratio. Daily rhythmicity was exhibited only by total proteins and albumin during the L/D cycle. We can claim that the fluctuation of serum total proteins and albumin concentrations are daily and not circadian and that they are driven by the L/D cycle.

Key words: total proteins; electrophoresis profile; daily rhythm; horse; photoperiod

INTRODUCTION

In clinical medicine the protein status of the organism is usually investigated by the assessment of serum/plasma total proteins. Total proteins are constituted by various fractions that are different

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among species. The two major fractions are albumin and globulins. Albumin is the most osmotically active serum protein used by many substances as principal carrier, and globulins are a heterogeneous group of proteins including vitamins, hormones, carriers of lipids, antibodies, and inflammatory, haemostatic and fibrinolytic molecules (Alberghina et al. 2010).

In physiological conditions total proteins levels could be influenced by many factors, such as age, body weight, hormones, pregnancy, lactation, oestrus condition, change in ambient temperature, or the nutritive state of the animal (Batavani et al. 2006). Moreover in mammals, a multitude of pathological conditions can cause changes in albumin and globulin concentrations. In horses, changes in concentrations of total proteins and their fractions could be attributed to poor performance, depression, fever, weight loss,

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diarrhoea, abdominal pain, and polyuria (Colhan et al. 1999).

Serum protein electrophoresis is the standard method of evaluating the concentrations of various protein fractions. As electrophoretic mobility differs among species, it has been essential to determine the "normal" electrophoretic pattern of each species in order to develop reference intervals that are used to identify abnormalities in patients.

One important factor usually ignored in the conventional "normal range" is the time of a biomedical measurement in relation to biological rhythms. In some cases, reference intervals determined irrespective of time are much too broad and indiscriminate. A "normal" value in conventional terms might be a false positive or a false negative, depending on when the measurement was performed in relation to a rhythm in the variable concerned. Alteration of one or several rhythm characteristics may reveal and quantify vulnerability or risk, prior to the occurrence of a given pre-disease or disease. So, the analysis of rhythm can show significant changes in parameters at determined times and it can give new information suggesting pre-symptomatic conditions (Tarquini et al. 1993). It is well known that haematological characteristics must be compared to the non-pathological reference values of the appropriate time of day, while several characteristics seem to be stable during the day since their circadian variations are not detectable or do not have any clinical significance (Berger 2004). For example, knowledge of the daily rhythm of serum creatinine in the dog allows us to predict progressive renal disease and the drugs treatment that could alter the kidney function (Giudice et al. 2009). In the same way, a better understanding of the circadian rhythm of the physiological functions of the eye such as tear production or intraocular pressure, could be useful in diagnosing a deficiency of the lachrymal system, corneal inflammation or damage that may lead to blindness (Piccione et al. 2008, 2009, Giannetto et al. 2009a, b). The circadian rhythms are most often described in terms of their phases and amplitudes, and how these respond, in both health and desease, to a single exposure to synchronisers (Berger 2008).

In literature little information is available about the circadian rhythm of total proteins and their fractions. The circadian variations of total protein, albumin, α_2 and γ -globulins have been observed in adult rats (Valli et al. 1979) but in horses and sheep, only the daily variation of total protein and albumin has been investigated (Piccione et al. 2005a). A different photoperiod has been observed to influence the values of total protein, albumin and α_2 -globulins, whereas it has been suggested that light is not the most important factor affecting α_1 - β and γ -globulins in the common vole (Dobrowolska and Gromadzka-Ostrowska 1983). On the other hand, seasonal changes in total serum proteins and their fractions in horses, have been connected with the changes of light and temperature throughout the year (Gill et al. 1985).

Because of the important role that serum proteins plays in the organism, it is undoubtedly useful to know their circadian rhythm; primarily, because they can affect the interpretation of the concentrations of the bound and unbound fractions of a physiological and pharmacological agent; and secondly because they should be taken into account in designing a therapeutic protocol that optimizes the tolerance and expected effects of drugs and diminishes their side effects.

The aim of this study was to investigate the daily rhythm of total proteins and their fractions in horses in order to establish if these rhythms are endogenously generated and to assess the role of light/dark cycle on these parameters.

MATERIALS AND METHODS

Five clinically healthy and regularly trained Italian Saddle horses (females, 6-10 years old, 530±20 kg body weight) were used in our study carried out in Messina, Italy (Latitude: 38°, 26' Longitude: 15°, 59'). All the horses were subjected to the same management regime and were housed in individual boxes $(3.00 \times 3.00 \text{ meters}, \text{ equipped with big})$ windows). Water was available ad libitum and hay and oats were provided 3 times a day (6:00; 12:00; 18:00). The animals were subjected to natural 13/11 Light/Dark (L/D) cycle (sunrise at 06:20 h, sunset at 19:20 h) and constant darkness (D/D). The D/D regime was the same as in the L/D cycle. Thermal and hygrometric records were carried out inside the box for the whole study by means of a data logger (Gemini, UK).

Blood samples (10 ml) were collected every 3 h over a period of 48 h, starting at 9:00 on day 1 and finishing at 9:00 on day 3, in vacutainer tubes without an anticoagulant (Terumo Corporation, Japan) via intravenous cannulas inserted into the jugular vein (MILA International, Florence, KY). The tubes were kept at room temperature for 20 min, then centrifuged at 3.000 rpm for 10 min and the serum obtained was stored at -25 °C until analysed. The concentration of serum total proteins was determined by the biuret method using an automated analyser UV Spectrophotometer (SEAC, Slim, Florence, Italy). The protein fractions were performed using an

automated system (Sel Vet 24, SELEO Enginering, Naples, Italy) according to the procedures described by the manufacturer. For each sample, 25 µl of serum were applied to numbered sample wells. Each holder accommodated up to 24 samples. The films were electrophoresed for 28 minutes at 450 V. After electrophoresis, the films were simultaneously fixed using an automated system, stained in red stain acid solution for 10 minutes, and then dried at 37 °C. After destaining in acetic acid and drying completely for 15 minutes the films were scanned on a densitometer, and electrophoretic curves plus related quantitative specific protein concentrations for each sample were displayed. Relative protein concentrations within each fraction were determined as the optical absorbance percentage, and absolute concentrations (g/l) were calculated using the total protein concentration. The major protein fractions were divided, according to the recommendation by the manufacturer, from cathode to anode as albumin α_1 , α_2 , β_1 , β_2 , γ -globulins, respectively. All housing and care conformed to the standard recommended by the Guide for the Care and Use of Laboratory Animals and Directive 86/609 CEE.

All results were expressed as mean \pm standard deviation (SD). Two-way repeated measures analysis of variance (ANOVA) was used to determine significant differences due to the time of day and experimental conditions on all parameters studied at the significant level $2\alpha = 0.05$. The data were analysed using the STATISTICA 8 (Stat Soft Inc., Tulsa, USA) software. Using cosinor rhythmometry (Nelson et al. 1979), four rhythmic parameters were determined: mesor (mean level), amplitude (half of the range of oscillation), acrophase (time of peak), and robustness (a stationary rhythm). The robustness of the rhythms was computed as the quotient of the variance associated with sinusoidal rhythmicity and the total variance of the time series (Refinetti 2004). Robustness greater than 40% is above noise level and indicates statistically significant rhythmicity.

RESULTS

During the experimental period, minimum and maximum temperatures were 21 and 26 °C respectively, and the relative humidity was 65%. The application of two-way ANOVA showed a significant effect of the time of day on total proteins, albumin, β_1 - and β_2 globulins and of experimental conditions on total proteins, α_1 -, α_2 -, β_1 -, β_2 - and γ -globulins (Table 1). No statistical modifications were observed on A/G ratio. The application of the periodic model and the statistical analysis of cosinor enabled us to define the periodic parameters and their acrophases during the 24 h of monitoring for both photoperiods.

Daily rhythmicity was exhibited only by total proteins and albumin during the L/D cycle. These rhythms are characterized by diurnal acrophases (16:28 and 17:20 respectively), amplitudes of rhythms of 0.50 for total proteins and 0.22 for albumin, and the robustness of rhythms were 48.2% for total proteins and 70% for albumin. Figs 1–2 show a mean pattern and mean values of four rhythmic parameters of serum total protein and albumin recorded during the experimental period.

DISCUSSION

Serum total proteins and their fraction in all the data points tested were within the physiological range suggested for horses (Kaneko et al. 1997), and all serum electrophoreses were characterized by the absence of a pre-albumin region and by six different bands: albumin, α_1 , α_2 , β_1 , β_2 , γ -globulins (Kohn 1957).

In contrast to the observations of Flisinska-Bojanowska et al. (1991), in which study serum total proteins showed no daily rhythmicity, but only small and insignificant changes during the day, our results reported a daily rhythmicity of serum total proteins and albumin during the L/D cycle.

The time series studied showed a central tendency of oscillation (mean level) of 64.9 and 33.5 for total protein and albumin respectively, that represent the point of balance of distribution of the studied parameters. The range of oscillation, which is twice this amplitude, identifies the boundaries of the oscillation. Therefore the amplitude of circadian rhythms are not necessary symmetrical so that the amplitude below the mean level may be different from the amplitude above the mean level. This condition is evident in Figs 1–2. The total protein range of oscillation is higher above the mean level than below. In contrast, the range of oscillation of the albumin is higher below the mean level than above.

Both rhythms were diurnal (Figs 1–2), their acrophases were similar, and were observed at 16:28 and 17:20 respectively for total proteins and albumin. This finding is challenged by a previous study conducted by Piccione et al. (2005a) in which nocturnal acrophases at the beginning of day (04:32) for total proteins and at the end of day (20:30) for albumin were observed. This finding also underlines the importance of exogenous factors – primarily the

Parameters (g/l)	Day 1 (L/D cycle) M±SEM	Day 2 (D/D cycle) M±SEM
Total proteins	64.8±1.49	65.9 ± 0.79
Albumin	33.4±0.60	33.8±0.27
α_1 -globulins	1.98 ± 0.09	1.72±0.13
α_2 -globulins	8.10±0.25	7.28±0.17
β_1 -globulins	6.78±0.40	7.91±0.27
β_2 -globulins	5.23±0.31	4.25±0.20
γ-globulins	9.32±0.37	10.78±0.34
A/G ratio	1.88 ± 0.30	10.81±0.31

Table 1. Serum total protein, protein fractions and A/G ratio recording during	g the ex	xperimental	period
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M = mean values, SEM = standard error of the mean





Fig. 1. Mean pattern of serum total protein recorded during day 1 (L/D) and 2 (D/D) in horses. Grey area represent the dark phase of the photoperiod. Dashed line indicates the Mesor value and Φ indicates the acrophase observed in day 1.



Fig. 2. Mean pattern of serum albumin recorded during day 1 (L/D) and 2 (D/D) in horses. Grey area represent the dark phase of the photoperiod. Dashed line indicates the Mesor value and Φ indicates the acrophase observed in day 1.

L/D cycle – and suggests that their circadian pattern is under L/D control, as previously observed on haematological parameters in this species (Piccione et al. 2005b), and excludes the influence of protein levels of the diet on daily rhythms (Greppi et al. 1996).

Even if the albumin daily rhythm was more robust than the serum total proteins daily rhythm, the influence of the first on the second rhythm is probably not linked to the concept that a rhythm with low robustness cannot be the cause of a rhythm with high robustness (Piccione et al. 2005a), but to the fact that the albumin constitutes about 50% of the serum total protein has a high impact on the serum total protein daily rhythm. Constant darkness is considered a "free running" circadian state. In the absence of temporal environmental cues the rhythm persists with a period approximately 24-hours. In this case the rhythm is defined as circadian. When the rhythm cycle is in 24 hour intervals and is not endogenously generated but susceptible by 24 hour environmental cycles it is called daily. Therefore, we can claim that the fluctuation of serum total proteins and albumin concentrations are daily and peak during the day and decrease in the morning during the natural L/D cycle. The results support the hypothesis that these rhythms are driven by an L/D cycle, considering that the other experimental conditions were constant through the study. Single samples from individuals are of little value for monitoring changes in total proteins and albumin due to pathological conditions; a series of measurements should be taken over a period of time, or samples should be collected at precise times for results to be meaningful. However, further studies are necessary to better understand the roles of light as a synchroniser of these rhythms.

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