ORIGINAL ARTICLE

Nycthemeral rhythms of total locomotor activity and oxidative markers in horse

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Summary

The aim of this study was to investigate the nycthemeral rhythm of total locomotor activity (TLA) in horse and the possible involvement of the daily organization of rest/activity cycles in the fluctuation of the redox state. For this purpose we recorded TLA and determined oxidative markers in ten clinically healthy Italian Saddle horses. TLA was continuously recorded by means of an actigraphy-based data logger (Actiwatch-Mini[®]). For the assessment of free radicals (dROMs), the antioxidant barrier (Oxy-ads) and the thiol-antioxydant barrier (SHp), blood samples were collected every 4 hours over a 48 h period. One-way repeated measures analysis of variance (ANOVA) showed a statistically significant effect of time of day on all studied parameters. The application of the periodic model and the statistical analysis of cosinor indicate, in horses, the existence of a daily rhythm of the studied parameters during the 48 h of monitoring. The results show that nycthemeral rhythms of TLA and oxidative markers have different trends in horse. dROMs and Oxy-ads showed a nycthemeral rhythm with an acrophase in the middle of the photophase, and the acrophase of SHp nycthemeral rhythm preceded them. In contrast, TLA showed its acrophase only after the middle of the photophase. TLA showed a lower robustness of rhythms (16.3 and 20.3%) and in respect to the robustness values of the rhythms of oxidative markers (67.3-86.2%). In conclusion, the results of the present investigation showed that oxidative markers have different patterns than locomotor activity, and further studies could be necessary to determine whether other external stimuli, such as solar radiation, food administration or physical exercise are able to influence redox state rhythms in this species.

Key words: nycthemeral rhythm; free radicals; horse; oxidative power; locomotor activity

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INTRODUCTION

In living beings numerous biological functions exhibit circadian rhythms whose generation is realised by a complex system with a central pacemaker located within the suprachiasmatic nuclei (Refinetti 2006, Berger 2008). Rapid progress in the elucidation of the mechanism of the circadian clock has been made over the last century. This has shown the circadian rhythmicity to be adaptations that allow organisms to prepare for relatively predictable events in their environment. The appearance of reproducible and stable circadian rhythms of high amplitude, and with a characteristic phasing with respect to other biological processes and the external environment, is believed to guarantee an optimal functioning of the biological system, with maximum efficiency, performance and wellbeing (Weinert and Waterhouse 2007). In fact, changes in the behavioural activity of animals are widely used as an indicator for the assessment of their welfare (Müller and Schrader 2003).

Among these indicators, the total locomotor activity (TLA) and the oxidative markers have received considerable attention. TLA has been documented in a large number of species of mammals (Piccione et al. 2010a). Most studies have been carried out on rodents, including laboratory and mole rats, domestic mice, hamsters, squirrels, voles and guinea pigs, but circadian patterns of TLA particularly, including different behaviour such as feeding, drinking, walking, grooming, ruminating, as well as all conscious and unconscious movements, have been well described in rabbits, cats, dogs, sheep (Piccione et al. 2006, 2007, 2010a, Refinetti 2006), goats (Piccione et al. 2008b, c) and horses (Bertolucci et al. 2008. Piccione et al. 2008a). The evaluation of total locomotor activity has been also studied in relation to the daily rhythms of the redox state in sheep (Piccione et al. 2010b) and dairy cattle (Giannetto et al. 2010).

Oxidative markers have also been documented, showing that more than one rhythm can be controlled by a single oscillator, and that multiple rhythms may be driven by different oscillators (Johnson 2001). In rats, for example, the circadian variations of the total antioxidant status are related to the circadian melatonin rhythm (Benot et al. 1998) whereas in humans it has been observed that free radical-scavenging activity is affected by physical activity and ingestive behaviours (Atsumi et al. 2008).

With this in mind, and considering the relevant interest in the redox state as a mediator of stress and pathological conditions and in TLA as an indicator for the assessment of animal welfare, the aim of this study was to investigate the nycthemeral rhythm of TLA in the horse and its possible influence on the fluctuations of free radicals and anti-oxidant power.

MATERIALS AND METHODS

Animals and housing

Ten Italian Saddle geldings (mean body weight 470 ± 30 kg, 7–9 years old) were used. Before the start

of the study, all subjects underwent a heart examination, respiratory auscultations, and routine haematology and plasma biochemistry. Only clinically healthy animals were used. Horses were kept in individual boxes under a natural photoperiod (12/12 LD cycle, sunrise at 06:00, sunset at 18:00) and a natural environmental temperature (18–21 °C; 60% relative humidity) in Sicily, Italy (latitude 37° 28' N, longitude 14° 37' E). Horses were fed *ad libitum* with hay (first cut meadow hay, sun cured, late cut 8 kg/horse/day) and a mix of cereals (oats and barley, 50% each, about 3.5 kg/horse/day, divided into two meals – 07:00 and 19:00). Water was available *ad libitum*.

All the treatment, housing and animal care reported above conformed to the standards recommended by the Guide for the care and use of animals (D.L. 27/1/1992, n 116) and EU (Directive 86/609/CEE).

Total locomotor activity recording

The total locomotor activity of horses, which includes behaviours such as feeding, drinking, walking, grooming and small movements during sleep, was recorded for two days of the experimental period. Each horse was equipped with an actigraphy-based data logger (Actiwatch-Mini[®], Cambridge Neurotechnology Ltd, UK), that recorded a digitally integrated measure of motor activity. This activity acquisition system is based on miniaturized accelerometer technologies, currently used for human activity monitoring, but also tested for activity monitoring in small non-human mammals (Muñoz-Delgrado et al. 2004, Mann et al. 2005). Actiwatch utilizes a piezo-electric accelerometer that is set up to record the integration of the intensity, amount and duration of movement in all directions. The corresponding voltage produced is converted and stored as an activity count in the memory unit of the Actiwatch. The maximum sampling frequency is 32Hz. Actigraphs were placed by means of collars that were accepted without any apparent disturbance. Activity was monitored with a sampling interval of 5 minutes. The total daily amount of activity, the amount of activity during the photophase and scotophase were calculated using Actiwatch Activity Analysis 5.06 (Cambridge Neurotechnology Ltd, UK). The Cosine peak of a rhythm (that is, the time of the daily peak) was computed by cosinor rhythmometry (Nelson et al. 1979) using the Actiwatch Activity Analysis 5.06 program.

Blood sampling

Blood samples (10 ml) were collected every 4 h over a 48 h period, starting at 8:00 on day 1 and finishing

at 8:00 on day 3, in vacutainer tubes without an anticoagulant (Terumo Corporation, Japan) via intravenous cannulas inserted into the jugular vein. Blood samples were centrifuged (ALC 4235 A Milan, Italy) at 3000g×20 min. The obtained serum was immediately analyzed by means of a UV spectrophotometer (model Slim SEAC, Firenze, Italy) for the assessment of the following parameters: reactive oxygen species (dROMs), antioxidant barrier (Oxv-adsorbent) and thiol antioxidant barrier (SHp). These techniques are based on the "spin traps" system, in which molecules react with free radicals, creating complexes revealed by spectrophotometry. The dROMs test is a colorimetric test that assesses the levels of hydroperoxides (R-OOH), the "markers" and "amplifiers" of tissue damage generated by peroxidation of lipids, amino acids, proteins and nucleic acids. In this test, these molecules, after reaction with a properly buffered chromogen, develop a coloured derivative, which is photometrically detected. The concentration of ROMs, which directly parallels changes in colour intensity, is expressed in Carratelli Units (1 CARR U=0.08 mg% hydrogen peroxide). Increased values directly correlate to increased levels of oxidative stress.

The oxy-adsorbent test evaluates the ability of plasma to oppose the massive oxidant action of an excess of hypochlorous acid in water solution by assessing photometrically the residual unreacted radicals of the acid. Decreased values directly correlate with the injury severity of "plasma barrier to oxidation". When the "excess" of radicals of hypochlorous acid after massive oxidation is high, the plasma barrier is reduced and vice versa.

The SHp test is a colorimetric determination of the plasma/serum thiol antioxidant barrier, which opposes peroxidative processes inhibiting both alkoxyl and hydroxyl radicals. This test is based on the ability of thiol groups to develop a coloured complex when reacted with DTNB (5,5-dithiobis-2-nitrobenzoic acid). The "titre" of thiols directly parallels colour intensity. Decreased values directly correlate with a lower efficacy of the thiols antioxidant barrier.

Statistical analysis

One-way repeated measures analysis of variance (ANOVA) was used to determine a statistically significant effect of time of day on total locomotor activity and oxidative markers at the significant level 2α =0.05. The data was analysed using the software STATISTICA 7 (StatSoft Inc., USA).

Using cosinor rhythmometry (Nelson et al. 1979), four rhythmic parameters were determined: mesor (mean level), amplitude (half the range of oscillation), acrophase (time of peak), and robustness (strength of rhythmicity). Rhythm robustness (stationarity of a rhythm) 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 10% is above noise level and indicates statistically significant rhythmicity.

RESULTS

One-way repeated measures analysis of variance showed a significant effect of time of day on all studied parameters on both days of monitoring.

The application of the periodic model and the statistical analysis of cosinor indicated the existence of a daily rhythm of the studied parameters in horse during 48 h of monitoring, and enabled us to define the periodic parameters and their acrophase during the two days of monitoring (Table 1). TLA and oxidative parameters showed a stable diurnal daily rhythm both of characterized by a different pattern (Figs 1-2). dROMs and Oxy-ads showed nycthemeral rhythms with acrophases in the middle of the photophase, and acrophase of SHp nycthemeral rhythm preceded them. In contrast, the TLA showed its acrophase only after the mid-point of the photophase. The TLA showed a lower robustness of rhythms (16.3 and 20.3%) in respect to robustness values of rhythms of oxidative markers (67.3-86.2%).

DISCUSSION

Our results showed that nycthemeral rhythms of TLA and oxidative markers have different trends in horse; this is not in agreement with previous studies in which the circadian oscillations of many behavioural processes and physiological parameters are paralleled (Langmesser and Albrecht 2006). These different patterns were accompanied by different robustness of rhythm values, and it is unlikely that a rhythm with low robustness might influence the rhythm with high robustness. Thus, the rhythm of TLA cannot be the cause of the redox state rhythm in horse. Whether the rhythm of activity is the cause of the other rhythms cannot be determined from the data on the robustness of this rhythm. Nevertheless, it was shown that the circadian organization of rest/activity cycles implies fluctuations in the level of free radicals oxygen species that are generated as by-products of the fluctuations in activity and metabolic rates (Langmesser and Albrecht 2006). So, the rhythmicity in radical formation should relate to that in oxygen

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Fig. 1. **Plexogram of horses kept in individual boxes under natural photoperiod** (12/12 LD cycle, sunrise at 06:00, sunset at 18:00) and natural environmental temperature. Locomotor activity is indicated by vertical grey marking. White and black bars indicate photophase and scotophase.



Fig. 2. Patterns of oxidative stress markers. Each point represents the mean of ten horses. White and black bars indicate photophase and scotophase.

Characteristics	Days of monitoring	Mesor	Amplitude	Acrophase (hours)	Robustness (%)
TLA	day 1	195.54	118.79	15:56	16.3
	day 2	206.22	133.77	16:37	20.3
dRoms (U Carr)	day 1	161.71	5.67	12:17	67.3
	day 2	159.71	5.87	11:32	85.7
Oxy-ads (µM)	day 1	438.22	7.54	11:43	76.2
	day 2	443.58	6.41	12:07	76.3
SHp (µM)	day 1	513.42	9.93	08:03	84.2
	day 2	511.79	11.22	08:39	86.2

Table 1. Mean values ± SD of four rhythmic parameters of total locomotor activity (TLA), free radicals (dRoms), antioxidant barrier (Oxy-ads) and thiol-antioxydant barrier (SHp), recorded during 48 hours of monitoring in horse.

consumption, which is widely documented in many animals and which should, in turn, depend on the circadian rhythms of locomotor activity (Hardeland et al. 2000).

Moreover, endogenous circadian and exogenously driven daily rhythms of antioxidative molecules have been described in various phylogenetically distant organisms. Substantial amplitudes were observed in several cases, suggesting the significance of rhythmicity in avoiding excessive oxidative stress (Hardeland et al. 2003).

Our finding, in which different variables exhibit different degrees of rhythmicity, is not surprising because it has been observed in other studies in which simultaneous recording of many variables was carried out (Refinetti 1999, Piccione et al. 2005, Giannetto and Piccione 2009). However, the finding is important because of its implications for the defense of the organism and in maintaining the redox state.

In conclusion, the results of the present investigation confirm that the monitoring of oxidative stress parameters contribute to the clinical evaluation of the horse but underline that oxidative markers are not affected by locomotor activity, and further studies are necessary to determine whether other external stimulus, such as solar radiation, food administration or physical exercise are able to influence redox state rhythms in this species.

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