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Full Research Article

SEASONAL DEVELOPMENT OF NOCTURNAL BLOOD PLASMA MELATONIN CONCENTRATION IN THE HUNGARIAN NATIVE DONKEY

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Abstract

The domestic donkey (*Equus asinus*) is a long-day, seasonally oestrous (polyoestrous) species, i.e., its reproduction is regulated by an endogenous circannual rhythm, similar to that of the horse (*Equus caballus*). In this, periodic melatonin production plays a major role, but little is known about its peripheral concentrations in the donkey. According to the authors' assumption, the cyclicity characteristic of melatonin concentrations inhorses may also be valid for donkeys. The sampling and statistical processing of the melatonin concentration made it possible to determine the nocturnal rhythm for special periods of the year (solstices, equinoxes) from the basic data. Blood samples were taken from 15 jennies during the year, covering the nocturnal half of the 24 h day.

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Blood plasma melatonin was assessed using the radioimmunoassay method. Corrected values were calculated for statistical processing. The range of corrected blood plasma melatonin concentrations was established as between 10 and 50 pg mL^{-1}. The midnight melatonin concentration (36.6 pg mL^{-1}) was significantly higher than that determined at 06:00 h and 18:00 h (24.6 and 24.1 pg mL^{-1}, respectively; $p<0.001$). The blood plasma melatonin concentration of 45.2 pg mL^{-1} at midnight on the winter solstice was the highest value determined under our geographical conditions. This value differed significantly from the melatonin concentrations at both the summer solstice and the spring equinox (34.2 and 30.7 pg mL^{-1}, respectively). However, the winter solstice value (45.2 pg mL^{-1}) did not differ significantly from the autumn solstice value (36.3 pg mL^{-1}). The results usefully expand the sparse pool of data gathered so far about the development of blood plasma melatonin concentration of domestic ass species.

Key Words: domestic ass, Northern Hemisphere, plasma melatonin, rhythmicity

INTRODUCTION

Melatonin regulates the daily and annual physiological cycles of the body, is influenced by the duration of light exposure, and its role is of paramount importance in reproductive processes in both animals (Kárpáti et al., 2023) and humans (Olcese, 2020). Light signals enter the hypothalamic suprachiasmatic nucleus via the retinohypothalamic nervebundle from the melanoprotective ganglion cells of the retina (Schroeder et al., 2018) and then reach the site of melatonin synthesis through the sympathetic noradrenergic pathway, the pineal gland (epiphysis). Light inhibits melatonin secretion, so the highest concentration of melatonin is measured in the dark.

Melatonin can be detected not only at its site of production, the pineal gland and, additionally, in the retina and placenta, but also in its extrapineal form in various organs. In the mitochondria of eukaryotic cells, melatonin provides protection against oxidative damage and contributes to the maintenance of cellular physiological processes. Melatonin modulates pituitary function and influences the release of prolactin, oxytocin and vasopressin. Furthermore, melatonin affects sexual development and stimulates the function of the hypothalamic-pituitary-ovarian (HPO) axis (Talpur et al., 2018). In the non-oestrous period, hypothalamic gonadotropin-releasing hormone (Sharp et al., 1987) and pituitary luteinizing hormone production (Hart et al., 1984) are low. The presence of melatonin is an essential factor in the persistence of the anoestrous period. The onset of the reproductive period is delayed by removal of the pineal gland (Grubaugh et al., 1982; Kilmer et al., 1982) or by cutting the sympathetic innervation of the gland (Sharp et al., 1979).

The domestic donkey (*Equus asinus*) is a long-day, seasonally oestrous (polyoestrous) species, i.e., its reproduction is regulated by an endogenous circannual rhythm, similar to that of the horse (*Equus caballus*). The main seasonal changes (winter-summer) have a significant impact on the rhythm's development, which is directly controlled by melatonin (Bartha et al., 2021). The main indirect regulator is the increased daylight in spring, which, as a seasonal change, reactivates the HPO axis and reduces melatonin concentrations (Nagy et al., 2000; Murphy, 2019). The natural, physiological reproductive period of the donkey in the northern hemisphere lasts from March to May, and its development is influenced by the lengthening of the day, temperature and energy content of the forage.

The production of melatonin is poorly understood in the donkey, and therefore,the goal of this research was to gain comprehensive knowledge about the rhythmicity of melatonin concentration in the blood plasma of domestic donkeys. We hypothesized that the cyclicity of the equine population in Central Europe may also be true for this species. The sampling and data processing allowed for detection of the nocturnal rhythms at selected times of the year (solstices, equinoxes).

MATERIALS AND METHODS

Description of the study population and sampling

Before the examination, the owners of the donkeys were informed about the purpose and publication of the research, which did not involve endangering the animals' welfare, and about the collection of data and blood samples from the animals. Donkey owners confirmed their participation by signing a written agreement. The project was approved under animal testing license PE/EA/01444-6/2022. The study was carried out in the Hungarian Native Donkey herd at Bőszénfa (Hungarian University of Agricultural and Life Sciences, Kaposvár Campus, Wildlife Landscape Centre), located at latitude 46.23° and longitude 17.85°. The average temperatures in December and June are 2 °C and 20 °C, respectively. The times of sunrise and sunset are 07:29 h and 16:05 h at the winter solstice and 04:56 h and 20:45 h at the summer solstice.

The domestic donkey is considered as a native species in our country. A breeding association was founded in 2003, and the studbook of the donkey population contains about 4000 individuals, with relatively few known ancestral lines. In the past, the use of donkeys in Hungary was mainly related to sheep herding and sheep farming (Ernst, 2004). The reasons for choosing to use donkeys in herding/farming were their easy handling and the reliable protection of the livestock entrusted to them. They were used as beasts of burden in rural farming, where their heat tolerance, coupled with a certain undemanding nature, made them particularly advantageous for keeping on low-quality pasture. The domestic population of Hungarian donkeys is not uniform in appearance or performance, lacking breed-specific traits compared to other European donkey breeds (Lénárt et al., 2017). The colour variation is reflected in differences in weight; grey animals have a significantly smaller body capacity than brown and black animals. Their average height at the withers is 117 cm, but this has a wide range. For achieving a homogeneous Hungarian domestic donkey breed, a breed reconstruction programme was started in 2021 (Harmat et al., 2022).

Animals in the study are kept under free-range conditions throughout the year. In winter, the donkeys also consume supplemental hay. They have constant access to drinking water. The health status of the stud is good. For this study, the condition of the animals was estimated visually and through palpation according to guidelines developed for donkeys (The Donkey Sanctuary). Condition scores ranged from 3 (ideal, in two thirds of observations) to 4 (overweight). Thus, the sampled animals were well or slightly overfed. The breeding season is limited to April and May. They are rebred in natural mating harems.

A day before the sampling, the animals were driven into their stable (laufstall) and kept there until the sampling was completed. During the harsh winter days, this barn provides shelter for them. Fifteen mares were selected for the study according to their pregnancy status; no other criteria were relevant in the selection and the same mares were retained for subsequent sampling. By mid-July, the pregnant mares had produced foetuses of about two months. Individual pregnancy status was determined at this time using a transrectal ultrasound scanner (Easy-Scan:Go, IMV Imaging Ltd., Bellshill, UK). Our preliminary processing (Harmat et al., 2024) confirmed that plasma melatonin concentrations in pregnant and empty mares did not show significantly different temporal variation from each other, and so pregnancy status was not included as a variable in the data processing for the current study. The median age of the mares was 9 years.

Sampling took place on three dates, assigned in advance: 14 July 2022, 12-13 November 2022 and 16-17 April 2023. Sampling occasions were over a 24-hour period, i.e. blood was taken approximately every hour and a half, always from a different individual. The light intensity in the barn was below 10 Lux at night, as determined by a light meter, and this value did not increase near the animals during sample collection.

Blood sample collection and determination of plasma melatonin concentration

Approximately 8 mL of blood were collected from the jugular vein into EDTA-containing vacuum tubes (Premium Vacuette® K3E K3EDTA Blood Collection Tube, Ref: 454086, Greiner Bio-One GmbH, Kremsmünster, Austria) at the time of sampling. The blood samples were then kept at 5 °C. Within one day, the blood samples were centrifuged at 2000 *g* using a universal centrifuge (Z 326 K, HERMLE Labortechnik GmbH, Wehingen, Germany) for 10 min to recover plasma. Plasma was collected in Eppendorf tubes and immediately frozen and stored at –18 °C until processing. For the *in vitro* quantification of blood plasma melatonin concentration (pg mL^{-1}) by radioimmunoassay, a Tecan RE29301 RIA kit (RE29301, IBL International GmbH, Hamburg, Germany) was used according to the manufacturer's guidelines. Each plasma was tested twice, and the mean melatonin concentration was taken as the individual, occasional result.

Statistical processing

In our study, we estimated individual melatonin concentrations for the time points of interest using population genetics. The matched sample data set we aimed for and the trends within it made this possible. The number of animals and samples and the statistical processing method ensure the reliable scientific value of the study. In repeated blood sampling studies such as our study, researchers are encouraged to reduce the number of animals used and to follow the 3Rs principle in their research.

For the processing of the melatonin results, the following factors were available:

– unique identification number; determined using a microchip reader,

– pedigree; the origin of the mares was known up to two ancestral lines,

– date of sampling; the day on which the blood was taken,

– time of sampling; hour and minute of blood collection,

– days around the winter solstice; number of days between 21 December 2022 and the date of sampling (ranged from – 160 to 128),

– minutes around midnight; time between midnight and the exact blood sampling time (in minutes, range ±540 minutes).

Statistical processing of the melatonin concentrations was performed in two steps. First, they were corrected by neutralizing the factors affecting them using an individual animal model (Pedigree Viewer, 2015). The factors considered were: sampling number (fixed effect), the additive genetic effect of the individual (random effect), and days around the winter solstice (as a covariate). The correction did not include the effect of minutes from midnight, because the runoff of melatonin concentration was evaluated as a function of this.

In the second step, the corrected melatonin concentrations were further adjusted for specific times of the year and time of day using linear regression. Thus, estimates were made for the summer and winter solstices and the autumn and spring equinoxes. For the times of day, the corrected values were calculated for 18:00h in the afternoon, midnight and 06:00 h in the morning.

One-way ANOVA with Tukey's honest significant difference test was applied to compare the melatonin concentrations for both year-specific days and time of day groupings. As a result, the mean and standard deviation are displayed. Statistica software (version 14; TIBCO Software Inc., 2020) was used for corrections and significance testing, as well as for graphical representation.

RESULTS

The range of blood plasma melatonin concentrations (corrected values) was between 10 and 50 pg mL^{-1} (Figure 1). The melatonin concentrations (adjusted) calculated for the time of day had a larger range (between 0 and

60 pg mL^{-1}), and the fitted second order curves show convincingly the increase and decrease of melatonin concentrations during the night hours. The blood plasma melatonin concentrations calculated for 18:00 h in the afternoon and 06:00 h in the morning were lower and practically identical. Estimates of melatonin concentrations in plasma at the time of the summer and winter solstices and the autumn and spring equinoxes also revealed circannual variations in melatonin concentrations. Blood plasma melatonin concentrations were highest at the winter solstice and lowest at the summer solstice.

The results of the statistical processing of melatonin concentrations in blood plasma, calculated for specific times of the day and seasons, are presented in Table 1. In this, the trends stated above are significantly confirmed. The overall mean was 24.6 pg mL^{-1} at 18:00 h in the afternoon, 36.6 pg mL^{-1} at midnight and 24.1 pg mL^{-1} at 06:00 h in the morning. Midnight melatonin concentrations were significantly (P<0.001) higher than concentrations both 6 h before and 6 h after midnight. There was no statistically proven difference between the latter.

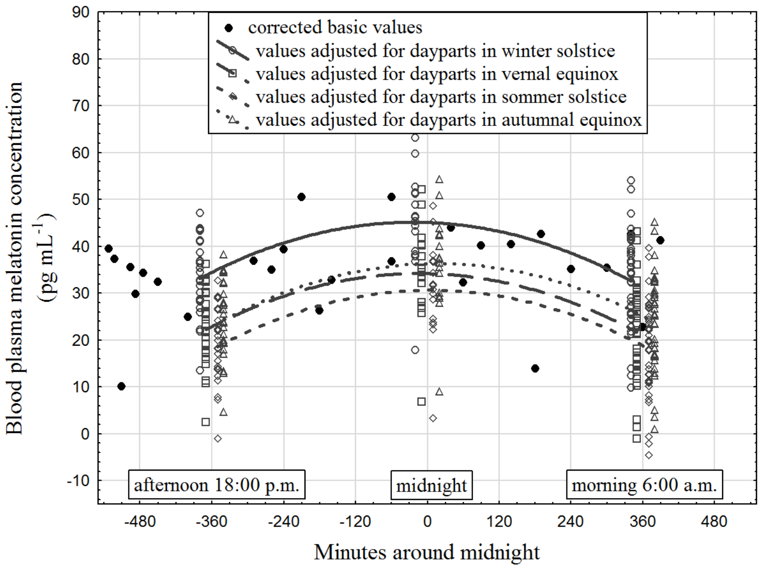

Figure 1: Development of blood plasma melatonin concentration of Hungarian native donkey mares calculated for specific times of the day and year, and its course by fitting quadratic equations

Table 1. Mean values of blood plasma melatonin concentrations (pg mL^{-1}) of Hungarian native donkey mares calculated for specific times of the year and day

Specific days of the year	Afternoon 18:00 h LSM1, SD2 P<0.001	Midnight LSM, SD P<0.001	Morning 06:00 h LSM, SD P<0.001
Summer solstice, 21-22 June 2022	18.7^{a} 8.03	30.7^{a} 10.15	18.1^{a} 10.18
Autumnal equinox, 23-24 September 2022	24.3^{a} 8.03	36.3^{a,b} 10.15	23.8^{a} 10.18
Winter solstice, 21-22 December 2022	33.2^{b} 8.03	45.2^{b} 10.15	32.6^{b} 10.18
Vernal equinox, 21-22 March 2023	22.2^{a} 8.03	34.2^{a} 10.15	21.7^{a} 10.18
Overall mean, P<0.001	24.6 9.57	36.6 11.28	24.1 11.42

LSM – least squares mean, **SD** – standard deviation
a,b – different superscript letters show significant differences in a column ($p < 0.05$)

The early darkening and late morning dawn in winter and the housing of mares in dark stalls during the studied times at the winter solstice caused a significant increase in melatonin concentrations, i.e., a significant difference from the blood plasma melatonin at the summer solstice and the spring and autumn equinoxes. A corrected blood plasma melatonin concentration of 45.2 pg mL^{-1} on the night of the winter solstice was the highest value found under our study conditions.

DISCUSSION

Our melatonin concentrationswere lower than the average autumn nocturnal melatonin concentration of 90 pg mL^{-1}obtained by Guillaume et al. (2006) in donkey mares. Thus, the melatonin concentration in the donkey is closer to that of the horse than was previously known (Altinsaat et al., 2009; Rapacz et al., 2010; Gáspárdy et al., 2023).

The previous partial observation by Guillaume et al. (2006) that melatonin concentrations increase in autumn is supported by our more complete findings. According to Cozzi et al. (1991), melatonin production in mules also shows cyclicity; higher melatonin concentrations appear in blood in autumn than in spring. However, the difference may also have a physiological background, as the intense decrease in melatonin concentrations at the beginning of the year is a precursor to the early reproductive period and the onset of oestrus in the long-day breeder donkey. The same phenomenon can be observed in our study donkey type. Also, Cozzi et al. (1991) showed that in mules, the afternoon increase in melatonin production was more seasonal than the morning decrease. In our study, we found a similar coherence, although this was not supported statistically; that is given the same 6 h time duration from midnight, afternoon melatonin concentrations were higher than morning ones.

CONCLUSION

The results obtained from the study of the domestic wild donkey significantly expand our previously fragmented knowledge about the development of melatonin concentration in the blood plasma of this species. We were the first to determine the changes in nocturnal melatonin concentration in the donkey species in Central Europe during the winter and summer solstices, as well as the spring and autumn equinoxes. Determining the rhythm of whole annual and daily or even within-oestrous cycle melatonin concentration in the donkey species requires further investigation.

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Authors' contributions

AG and SGyF conceived and supervized the study and made considerable efforts in the completion of manuscript. LH and JN participated in the collection of the blood samples and background information for data processing and writing a first draft of the paper supplemented with ANC and ZsB. BS and AA carried out the radioimmunoassay and contributed to the processing of literary sources. AG had significant role in statistical data analysis. LH and ZsB finalized the manuscript for submission.

Competing interests

The authors declare that they have no competing interests.

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SEZONALNE PROMENE NOĆNE KONCENTRACIJE MELATONINA U KRVNOJ PLAZMI DOMAĆEG MAGARCA U MAĐARSKOJ

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Kratak sadržaj

Domaći magarac (*Equus asinus*) je, slično domaćem konju (*Equus caballus*), sezonski poliestrična životinja tokom dela godine sa dugim trajanjem dnevnog fotoperioda sa endogenim godišnjim ritmom reprodukcije. Ipak, veoma se malo zna o promenama koncentracije melatonina u krvnoj plazmi magaraca.

Prema pretpostavkama autora cikličnost koja je karakteristična za konje eventualno može da se odnosi i na ovu vrstu. U cilju proveravanja ove sumnje uzorkovanje i statistička obrada podataka je vršena na takav način da dā uvid u noćni ritam pri posebnim periodima godine (solsticiji, ravnodnevnice).

Krv je uzorkovana od 15 magarica tokom jedne godine u noćnoj polovini dana. Koncentracija melatonina u uzorcima je određena radioimunološkom analizom. Izračunete su korigovane vrednosti za statističku analizu.

Opseg metode za određivanje koncentracije melatonina je od 10 do 50 pg mL^{-1}u odnosu na korigovane vrednosti. Ponoćna koncentracija melatonina (36,6 pg mL^{-1}) je značajno veća od vrednosti izmerenih u 6:00 ujutru, odnosno 18:00 uveče (24,6 i 24,1 pg mL^{-1}, za redom; $P<0,001$).

Vrednost od 45,2 pg mL^{-1} u ponoći zimskog solsticija predstavlja najviši nivo melatonina određen na našem geografskom području. Ova vrednost značajno se razlikuje kako od one izmerene tokom letnjeg solsticija tako i od one dobijene tokom prolećne ravnodnevnice (34,2 i 30,7 pg mL^{-1}, za redom). Ipak, odstupanje od vrednosti jesenje ravnodnevnice statistički nije značajno (36,3 pg mL^{-1}).

Dobijeni rezultati značajno doprinesu proširenju malobrojnih podataka koji do sada bili dostupni o promenama koncentracije melatonina u krvnoj plazmi domaćeg magarca.

Ključne reči: domaći magarac, severna hemisfera, melatonin u plazmi, ritmičnost