

The possible role of human milk nucleotides as sleep inducers

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Breast-milk contains a potent mixture of diverse components, such as the non-protein nitrogen fraction which includes nucleotides, whose variation in levels is evident throughout lactation. In addition, these substances play an important role in sleep homeostasis. In the present study, human milk samples were analyzed using a capillary electrophoresis system. The rhythmicity of each nucleotide was studied by cosinor analysis. It was found that the nucleotides 5'AMP, 5'GMP, 5'CMP, and 5'IMP have significant ($P < 0.05$) circadian rhythms, the acrophases of the first two being during the night, and of the latter two during the day. While 5'UMP did not show a clear circadian rhythm, there was an increase in its levels at night. In conclusion, the rise in nocturnal levels of 5'AMP, 5'GMP, and 5'UMP could be involved in inducing the 'hypnotic' action of breast-milk at night in the infant.

Keywords: nucleotides, circadian, sleep, human milk, capillary electrophoresis

Introduction

A joint declaration by the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) stated that breast-milk is the optimal food for infants and can never be equalled by artificial substitutes. It covers all the child's physiological and nutritional needs during the first 4–6 months of life.¹ For this reason, there is growing interest in attempting to make infant formulas that more closely resemble mother's milk. Infant formulas are the only processed food products that fully meet the nutritional needs of infants during the first months of life until the introduction of adequate supplementary feeding.²

The milk of every mammalian species has a different composition, tailored to the digestive, nutritional, and growth needs of its offspring. Human milk is a living fluid that changes with time, with its composition and volume being modified both during the course of each day and throughout the breast-feeding period. Its non-protein nitrogen fraction includes nucleotides whose concentrations are known to vary throughout lactation. In particular, there is an increase in nucleotide concentration in the mature milk (from day 15 postpartum) relative to the colostrum (4–5 days' postpartum).³

Nucleotides are the building blocks of nucleic acids responsible for storing and transmitting genetic information. They are precursors of energy-rich compounds that control the metabolic processes (biosynthesis, fundamentally) in all cells. Their skeleton consists of a pentose (carbohydrate), a nitrogen-containing base, and a phosphate group. The commonest are nucleotides where the nitrogen-containing base is a purine – adenosine

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5'monophosphate (5'AMP), guanosine 5'monophosphate (5'GMP), and their precursor, inosine 5'monophosphate (5'IMP) – or a pyrimidine – uridine 5'monophosphate (5'UMP), cytidine 5'monophosphate (5'CMP), and thymidine 5'monophosphate (5'TMP).

The nucleotides act in cells as secondary messengers through cAMP (cyclic 5'AMP) and cGMP (cyclic 5'GMP), and also supply the necessary chemical energy. They can also act as components of many enzyme co-factors such as flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD), in addition to having a strong influence on sleep – the function which is the objective of the present study.

In reviewing the literature, we found that three nucleotides are considered to be involved in the physiological function of sleep – 5'UMP, 5'AMP, and 5'GMP.

The first, 5'UMP, is distributed throughout the body (including the brain), and has a depressive effect on the CNS. The nightly administration of low doses of this nucleotide produces a moderate increase in the number of REM and non-REM sleep episodes,⁴ but has little or no influence on their duration.^{5,6} The plasma concentration of uridine in mice has a marked circadian rhythm,⁶ with the time of the maximum concentration (acrophase) coinciding with the time of least activity.

The second, 5'AMP, is the nucleotide which is most referred to in the literature as a sleep inducer. Indeed, its hypnotic properties have been recognized now for over 30 years.⁷ More recent evidence confirming its role in sleep induction is based on several facts: extracellular concentrations (through the secondary messenger cAMP) present circadian variations, its administration induces an hypnotic effect, and its levels decline during the period of wakefulness.^{8–11}

The third, 5'GMP, is also a second messenger in its cyclic form (cGMP), which mediates most of the neuronal effects of nitric oxide (NO). Many studies have pointed to the role of NO in sedation. For instance, the injection of a cGMP inhibitor into rats was found to increase wakefulness at the same time as suppressing REM and non-REM sleep.¹² Human studies have shown that cGMP plasma concentrations rise when the subject goes to bed and remain high throughout the night, reflecting its role in stimulating the secretion of the pineal hormone melatonin.^{13,14}

Recently there has been growing interest in studying nucleotides in the diet, since they seem to play an important role in human nutrition at different stages of life. This is especially so in infancy, as they influence neonatal development by the synthesis of

phospholipids, by modifying the microflora and repairing any damage in the gut, and also by participating in the T-lymphocyte mediated immune response.^{15–18} It has been suggested that both the nucleotides and the nucleosides found in human milk may be important for tissue development in infants.¹⁹

The Co-ordinated International Expert Group of the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) recommends the following maximum concentrations for nucleotides added to infant formulas: 1.75 mg/100 kcal of 5'CMP, 1.5 mg/100 kcal of 5'UMP, 1.5 mg/100 kcal of 5'AMP, 0.5 mg/100 kcal of 5'GMP, and 1 mg/100 kcal of 5'IMP. Also, the total of all nucleotides must not exceed 5 mg/100 kcal.²⁰

Although the most extensively validated method for nucleotide assay in human milk is high performance liquid chromatography (HPLC), our research group has demonstrated that capillary electrophoresis (CE) is another perfectly viable technique.²¹ Nearly all nucleotide determinations have studied the variations in their concentrations over the months of lactation. The novelty of the present work is the study of the possible circadian rhythms in the nucleotide content of breast-milk by determining the changes that occur during each 24-h period. This is essentially the reason for using CE as against HPLC, since measurements with CE are significantly faster (approximately 30 min compared with 2 h in HPLC). Also, the efficiency of the method is much greater (more than 200,000 plates theoretically, compared with 5000 for HPLC), and the expenditure in terms of the volumes of reagents and samples is much lower.

Subjects and methods

Subjects

The study population consisted of 30 healthy mothers from the region of Extremadura (Spain) who had been breast-feeding for 3 months. Their median age was 33 years (minimum-to-maximum range, 26–39 years), and the mean \pm SD values for weight, height, and body mass index (BMI) were 62.3 ± 7.3 kg, 164 ± 6 cm, and 23.1 ± 2.4 kg/m², respectively. The subjects were considered healthy on the basis of their breast-feeding success, a physical examination, and a follow-up. All subjects were informed about the investigation, and gave their written consent.

During the study, the subjects took no drugs that would disturb the levels of nucleotides. The Ethical Investigation Committee of University of Extremadura approved the study.

Samples

Samples of breast-milk were collected in polystyrene tubes before each feed over a 24-h period, during March to July, and stored frozen at -30°C until assay in duplicate. In general, between 6 and 8 samples of breast-milk were obtained from each mother.

Equipment and components

The CE system used was a P/ACE MDQ System 5510 equipped with a diode array detector (Beckman Coulter, Inc., USA). The system can be rapidly reconfigured from a flexible research platform to a tightly regulated routine-use platform. Automated fractionation of a detected peak allows isolation of newly resolved compounds for external identification.

Capillary cartridges

The capillaries are housed in user-assembled cartridges which are compatible with all current CE capillaries. For the present study of nucleotides, the CE separations were carried out in an uncoated silica capillary ($75\ \mu\text{m}$ i.d. \times $375\ \mu\text{m}$ o.d.; Polymicro Technologies[®], LLC, USA) with an effective length of 20 cm.

Detector modules

To allow for flexible method development and rugged routine use, the design of the P/ACE MDQ makes it easy to interchange high-sensitivity diode array (DAD), UV/Vis, and laser-induced fluorescence (LIF) detection modules. An external detector adapter allows the capillary to be extended to additional detection systems.

Software for the CE analysis

The 32Karat[™] software package specific to capillary electrophoresis includes mobility plot generation, advanced reports, and new 2-D algorithms to couple mobility and spectral signatures for peak identification. All of this results in a fully integrated CE control and data analysis workstation.

The methods are defined and edited in table format. All functions for the system are handled in a single window, including programming of the buffer array for the automation of strategies for the development of methods, using filters such as scan range, wavelength maximum, and mobility.

Control and analysis

Peak identification using either time or mobility, coupled with spectral signature confirmation, creates powerful 2-D peak identification schemes.

Velocity-calibrated peak area and CAESAR[®] integration ensure reproducible quantification at low limits of detection.

Reagents

Adenosine 5′monophosphate, uridine 5′monophosphate, guanosine 5′monophosphate, thymidine 5′monophosphate, cytidine 5′monophosphate, inosine 5′monophosphate, boric acid, and sodium dodecylsulphate (SDS) were purchased from Sigma-Aldrich (USA). All other chemicals were of analytical purity grade. Perchloric acid 60%, sodium hydroxide and potassium hydroxide 85% pellets were purchased from Panreac, Spain. All solutions were prepared using deionized water (Milli-Q System).

Procedures

Preparation of stock solutions

The values reported in the literature indicated that the nucleotide concentrations in human milk would be in the range 0–9 $\mu\text{g}/\text{ml}$. Stock nucleotide solutions were, therefore, prepared in the following concentrations: 10, 5, 1, and 0.5 $\mu\text{g}/\text{ml}$ of 5′AMP, 5′CMP, 5′GMP, 5′TMP, 5′UMP, and 5′IMP.

Extraction of nucleotides from breast-milk

We followed the technique of Perrin *et al.*²² with certain modifications. We started from milk samples of healthy women of at least 12 weeks' lactation. Aliquots of 0.75 ml of each sample were hydrolysed with 0.75 ml of 13% perchloric acid, mixing for 45 min on a roller mixer. After centrifuging at 5000 g for 20 min at room temperature, the supernatant was collected, discarding the fatty halo.

The solution was then adjusted to neutral pH with 5 M KOH, and left in an ice bath for 1 h for all the potassium perchlorate to precipitate. It was then filtered through a 0.45 μm membrane filter (Millex; Millipore, USA) before assay.

CE analysis

All experiments were performed on a P/ACE System 5510 (Beckman Coulter). The CE separations were carried out in an uncoated silica capillary ($75\ \mu\text{m}$ i.d. \times $375\ \mu\text{m}$ o.d.; Polymicro Technologies) with an effective length of 20 cm. Detection was by UV light over the range 190–300 nm (cartridge detection window $100 \times 800\ \mu\text{m}$) and the limit of detection (LOD) was 60 ng/ml.

Samples were loaded by low-pressure injection (3.45 kPa) for 6 s (14.3 nl, 2.7% of the total capillary volume injected). Borate buffers were prepared from boric acid, then SDS was added, and the solution was adjusted with 500 g/l NaOH to the appropriate pH. The capillary was washed at the beginning of each working day with deionized water, 0.1 M sodium hydroxide, water, and finally with a separation buffer for 5 min.

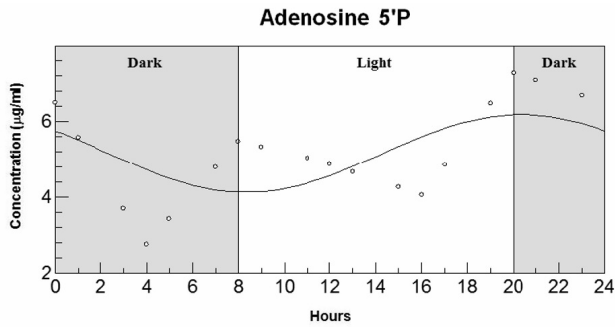


Figure 1 Sinusoidal function obtained by cosinor analysis of the nucleotide 5'AMP for a 24-h period ($n = 30$)

Between runs, it was rinsed with water for 1 min and with a separation buffer for 2 min. The assays were run at constant voltage using a ramp of 1 min. The alkaline (borate) separation system as described by Adam *et al.*²³ was used as follows. The capillary was operated at 30°C. The separation buffer was prepared from boric acid (60 mmol/l), SDS (80 mmol/l), and adjusted with 2-amino-2-methyl-1-propanol to neutral pH. Assays were run at +10 kV (positive outlet). The detector's data rate was set at 4 Hz.

Chronobiological analysis

The chronobiological analysis of the data was performed using Ritme® for Windows software package. The rhythmicity of each nucleotide was studied by cosinor analysis.²⁴ The sinusoidal function used for the fit is the following:

$$y(t) = M + A \times \cos [(2 \times \pi/\tau) \times t - \Phi] \quad \text{Eq. 1}$$

where $y(t)$ is the value of the cosine function at time t , M is the mean level of oscillation or the MESOR (acronym of midline-estimating statistic of rhythm, the mean value about which the oscillation occurs, equal to the arithmetic mean of equidistant data covering a whole number of cycles), A is the amplitude (measure of the extent of a rhythmic change in a cycle as estimated by the sinusoidal function that best fits the data), the frequency ($\omega = 2 \times \pi/\tau$) where π is the number pi and τ is the period (24 h in our case), and Φ is the acrophase (a phase angle measuring the timing of the peak activity, expressed as the lag from a reference time to the crest time of the best fit sinusoidal function). Therefore, cosinor analysis determines the best-fitting sinusoidal wave by estimating three parameters – mesor, amplitude, and acrophase.

Sample distribution

Given that the times at which milk samples were extracted did not exactly coincide from one mother to another, we selected those hours of the 24-h period for

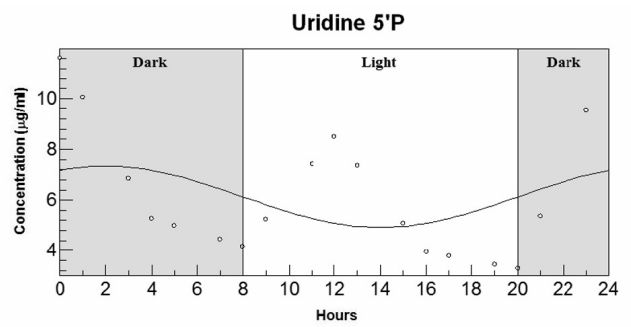


Figure 2 Sinusoidal function obtained by cosinor analysis of the nucleotide 5'UMP for a 24-h period ($n = 30$)

which there were the greatest numbers of samples under the constraint of requiring reasonably uniform distribution of those hours.

By cosinor analysis, we determined the confidence limits of the MESOR, amplitude, and acrophase at 95% probability level. When the range determined by the confidence limits of the amplitude contains the value 0, it cannot be excluded that the amplitude is 0 and, therefore, the existence of a rhythm is not statistically significant. In other words, to test the statistical significance of the rhythm, we determined whether the null hypothesis of zero amplitude is or is not rejected at 0.05 of alpha level. The P -value indicates the significance of the fit of the cosine curve to the data.

The confidence limits of the acrophase allow one to determine whether there were significant differences between the acrophases of different variables. When the range determined by the confidence limits of the acrophase of one variable overlaps that of another, the possibility that both acrophases are equal cannot be discarded.²⁵

Results

Figure 1 shows the levels of 5'AMP in human milk over a 24-h period. The levels increase as night falls (after 20:00), and the levels are higher at the first hours of the night relative to the interval before dawn.

Figure 2 shows the equivalent results for 5'UMP. In this case, there was an increase in the middle of the night with respect to the previous hours and with respect to the light hours.

Figures 3–6 present the results for the other four nucleotides (5'GMP, 5'CMP, 5'IMP, and 5'TMP) in which variations between the different time hours showed no difference. In Figure 3, however, there was an apparent increasing trend of the levels of 5'GMP for the nocturnal period (20:00–08:00). A similar

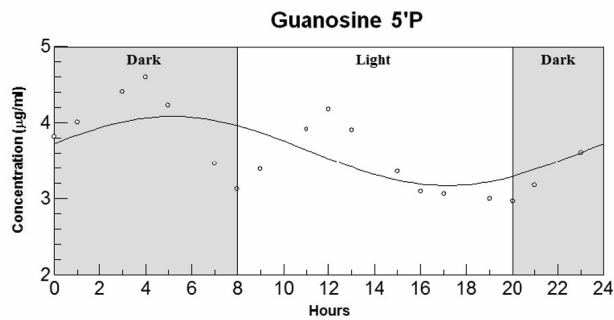


Figure 3 Sinusoidal function obtained by cosinor analysis of the nucleotide 5'GMP for a 24-h period ($n = 30$)

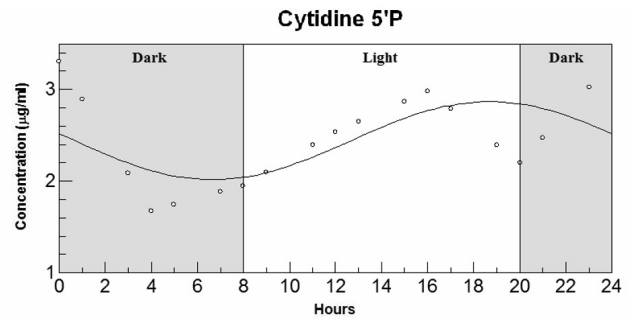


Figure 4 Sinusoidal function obtained by cosinor analysis of the nucleotide 5'CMP for a 24-h period ($n = 30$)

trend, but during daylight hours (08:00–20:00), is observed for 5'CMP and 5'IMP (Figs 4 and 5, respectively). This contrasts with the apparent downward trend in the daylight intervals for 5'AMP (Fig. 1).

The results of the chronobiological study (Table 1) of particular interest were the significant circadian rhythms of 5'AMP (Fig. 1) and 5'GMP (Fig. 3) with acrophases during the period of darkness (at 20:19 and 05:08, respectively). The other two nucleotides having significant circadian rhythms were 5'CMP (Fig. 4) and 5'IMP (Fig. 5) but with acrophases during the daytime period (at 18:40 and 19:14, respectively).

Discussion

Breast-milk is not static in its composition, but changes with time,^{26,27} in parallel with the infant's energy demands and tissue growth. For the newborn, there is an accentuated protein demand because of the anabolic requirement involved in the first weeks of growth.

Nonetheless, there has until now been no consideration of the possibility that, through her milk, the mother is preparing her baby's adaptation to the changing environment – day and night, for example. It is now known that high levels of melatonin in breast-milk appear during the night and low levels during the day.²⁸ Since melatonin is the hormone that regulates the

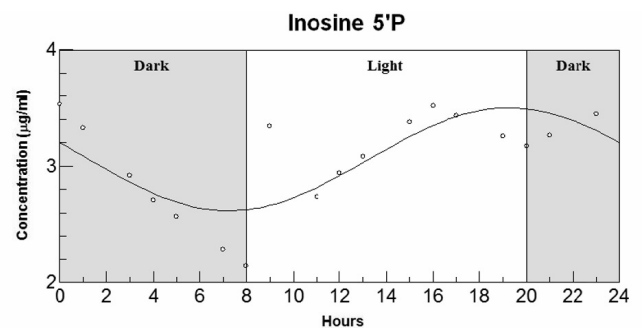


Figure 5 Sinusoidal function obtained by cosinor analysis of the nucleotide 5'IMP for a 24-h period ($n = 30$)

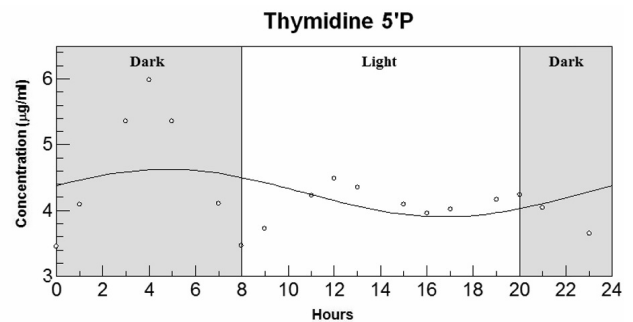


Figure 6 Sinusoidal function obtained by cosinor analysis of the nucleotide 5'TMP for a 24-h period ($n = 30$)

sleep/wake cycle, these changes in breast-milk will doubtless be the signal to help the baby adapt as quickly

Table 1 Chronobiological parameters of each nucleotide for a 24-h period

Nucleotide	MESOR (µg/ml)	Amplitude (µg/ml)	Acrophase (h:min)	Cosinor significance <i>P</i> -value
5'AMP	5.17 (4.61–5.73)	1.03 (0.02–2.03)	20:19 (15:08–25:30)	0.04457*
5'UMP	6.14 (4.91–7.37)	1.20 (–)	02:00 (–)	0.36425
5'GMP	3.63 (3.42–3.85)	0.46 (0.07–0.84)	05:08 (01:18–08:58)	0.01955*
5'CMP	2.44 (2.25–2.64)	0.42 (0.16–0.68)	18:40 (14:59–22:20)	0.01645*
5'IMP	3.06 (2.91–3.21)	0.44 (0.18–0.70)	19:14 (16:48–21:41)	0.00149*
5'TMP	4.27 (3.94–4.60)	0.36 (–)	04:45 (–)	0.28860

MESOR values and amplitudes are in the corresponding parameter units. Acrophases are given as times of day (08:00–20:00 light/dark cycle). Confidence limits are in parentheses. The *P*-value indicates significance of the fit of the cosine curve to the data. **P* < 0.05 was considered statistically significant ($n = 30$).

as possible to the day/night versus sleep/wakefulness environment.^{29–31}

The present study continues this line of inquiry into the change and temporal evolution of the macro- and micro-nutrients in breast-milk. Our purpose was to study some of the possible variations, but on a much shorter time scale, in particular the 24-h period variation of the nucleotides belonging to the non-protein nitrogen fraction. As was first described some 30 years ago and has been confirmed in recent years, these nucleotides have a great genetic importance³² via their action on the flora in the gut,¹⁵ and neurochemically via their intracellular action as secondary messengers, particularly the physiological action of the purine nucleotides on sleep.⁸ Also, in the last few years, their hypnotic action in infants has been demonstrated by the results of applied research with starter milks for infants with sleep problems.^{21,30,31}

The higher nocturnal levels of the purine nucleotide 5'AMP were consistent with its nature as a sleep inducer as found in earlier studies.^{33–35} In addition, as a novel result compared to those reported by other workers,³⁶ we demonstrated the existence of a circadian rhythm for this nucleotide. The increase was confined to the beginning of the night (with acrophase at 20:19, and a MESOR of 5.17 µg/ml), and could mean that the cAMP which is used in the release of GABA, an inhibitory and 'sleep-promoting' neurotransmitter,³⁷ originates from this nucleotide in the milk. It is notable that the increase of this nucleotide coincides with the onset of darkness at 20:00, and that the raised levels are maintained over a long time to conserve the cAMP-mediated intracellular response, especially in brain tissue in order to maintain homeostasis during sleep.³⁴

The other purine nucleotide, 5'GMP, showed a tendency to increase during the night, unlike the periods of daylight during which its levels were more irregular. This nucleotide is a precursor of another intracellular messenger (cGMP) which, during the night, is involved in the secretion of the hormone melatonin, thereby inducing and entraining nocturnal rest.^{13,38,39} Our chronobiological study showed this nucleotide to have a clear circadian rhythm, with the acrophase in the final hours of darkness, at 05:08 (an acrophase that is very similar to that reported by Skala *et al.*³⁶) and a MESOR of 3.63 µg/ml.

With respect to 5'UMP, this nucleotide did not describe a clear circadian rhythm, but its concentrations gradually decreased during the hours of daylight, followed by a clear increasing trend during the period of darkness, indicating a possible ultradian rhythm, which is understood as being part of the

stimulation and functioning of the hypnotic mechanism.^{4–6,40}

Of the other nucleotides, 5'CMP had a significant circadian rhythm with acrophase at 18:40 (during daylight hours), and a MESOR of 2.44 µg/ml. Because 5'IMP is the precursor of the other two purine nucleotides, it was not surprising that it showed a significant circadian rhythm that was in synchrony with the other two purine nucleotides, 5'AMP and 5'GMP. Indeed, its acrophase was at 19:14 (just before the onset of darkness when the sleep inducers, 5'AMP and 5'GMP, reach their acrophases) and its MESOR was 3.06 µg/ml.

Conclusions

The assay of nucleotides in the breast-milk of the study population showed that their levels were not constant over a 24-h period. This was particularly so for 5'AMP, 5'UMP, and 5'GMP, which showed increased concentrations at night and may, therefore, be involved in inducing hypnotic action in the infant.

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