The Effect of Non-nutritive Sucking on Expression of *BMAL1* and *CRY1* in Peripheral Blood Mononuclear Cells of Preterm Neonates: A Preliminary Study

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Abstract

Objectives: To determine whether non-nutritive sucking (NNS) has an effect on circadian rhythm of circadian genes in peripheral blood mononuclear cells (PBMC) in preterm neonates. ***Methods:*** Ten premature neonates, gestational age from 30 to 31 weeks, were recruited. Infants with birth asphyxia, respiratory distress syndrome, apnoea, malformation, infection or haemolytic diseases were excluded. All infants were provided with fifteen minutes of NNS every three hours before a tube feed over a period of two weeks. At 7am and 7pm on the 1st day, 7th day and 14th day of hospitalisation, peripheral venous blood was obtained from the premature babies and real-time reverse transcription polymerase chain reaction was used to determine the expressions of *BMAL1* and *CRY1*. ***Results:*** In this study, preterm infants treated with NNS, the expressions of *BMAL1* and *CRY1* in peripheral blood mononuclear cells did not have a statistical difference between 7am and 7pm on the 1st day, 7th day or 14th day of hospitalisation. ***Conclusions:*** The study results suggested that NNS did not affect *BMAL1* and *CRY1* gene expressions in PBMC of preterm neonates.

Key words   Circadian; Non-nutritive sucking; Neonatal intensive care

Introduction

Preterm neonates have difficulty adjusting to the extra-uterine environment. They come out from a warm and comfortable intrauterine environment to a relatively cold and noisy outside world, they have to cope with many new physical needs: such as respiration, eating, keeping warm, etc. Although neonatologists do their best to help them, many problems may still appear later.

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Circadian rhythm regulates the biochemical and physiological processes in the body including energy metabolism, gastrointestinal tract movement, sleep-wake cycles, cardiovascular activity and endocrine secretion. Researchers have found that the circadian system develops prenatally and progressively matures after birth.[1] If preterm infants can be induced to sustain the circadian rhythm in outside environment it might be easier for them to adapt to the outside world.

Recently two circadian rhythm pacemakers[2] have been identified. One is in the suprachiasmatic nucleus (SCN), which is already well known as a light entrainable oscillator. At the molecular level, mammalian circadian clocks are based on cellular oscillators built from a set of clock genes organised in interlocked transcriptional feedback loops. The *CLOCK* and *BMAL1* drive expressions of two *CRY* and three *PER* genes via E-box. *PER* and *CRY* proteins negatively feed-back on *CLOCK/BMAL1* activity, thereby generating a stable 24-hour rhythm of transcriptional activity. The other pacemaker is the food entrainable

oscillator, which is located outside the SCN, however, its exact location is not certain.[3-5] In rodents, cyclical feeding has been shown to affect the circadian rhythm and this non photic entrainment was characterised by food anticipatory activity (FAA). There is evidence that rats with complete SCN lesions continued to exhibit anticipation to temporally restricted feeding. *In vivo*, most circadian oscillators outside SCN are preferentially entrained by feeding time. Circadian gene rhythms in stomach, intestines, pancreas, liver, adrenal gland, lungs, heart and other tissues are shifted to realign with the daily rhythm of food intake.[6] Intrinsic periods of FAA rhythms of mice are affected by *CRY* deficiency. *BMAL1*(-/-) mice exhibits a pre-feeding increase of activity far from circadian range, indicating a deficit in circadian oscillation.[7] According to current theory of light entrainable oscillator, because *BMAL1* and *CRY1* are the opposite genes of the positive and negative regulators of the transcriptional-translational feedback loops, we therefore choose them as the target genes in our study.

Non-nutritive sucking (NNS) is a method that keeps infants calm and quiet. It minimises pain sensation in infants. It reduces the length of their hospital stay, decreases time in fussy and awake states and makes them exhibit less defensive behaviours.[8,9] It assists neurodevelopmental organisation, aids neurobehavioural maturation and it may also optimise ventilation in preterm infants who require nasal non-invasive ventilatory support.[10] It is used to preserve infants' sleep and reduce agitation during painful procedures and effectively reduces pain scores more than routine care during heel-stick procedures.[11,12] Non-nutritive interventions for premature infants who receive tube feeds may enhance the transition from tube to oral feeding by allowing the infant to practice using their oral motor musculature.[13] NNS associated with oral stimulation programs could improve breastfeeding rates among preterm infants with very low birth weight.[14] NNS also helps insulin release into the gastric lumen in preterm infants,[15] and it may also decrease the risk of sudden infant death syndrome.[16] For preterm neonates gestational age from 30 to 31 weeks, during their first two weeks, the coordination necessary for suck-swallow-respiration to support safe oral feeding is likely underdeveloped. They are usually fed by a nasal tube with formula-milk injected into their stomachs. The infants are passively gavage-fed, but they could be induced to suck a silica gel nipple; which is called NNS. NNS increases gastric motility and the infant is called into active oral movements. Based on this evidence we suppose that NNS would act as a food entraining cue and thereby influence circadian rhythms.

In adult humans, circadian clock genes are expressed in a circadian manner in peripheral blood mononuclear cells (PBMC).[17] Our former report indicated there was no circadian fluctuation of circadian genes *BMAL1* and *CRY1* in PBMC of preterm infants.[18] It is unknown whether NNS would have any stimulatory effect on the circadian genes *BMAL1* and *CRY1*. If their expressions are demonstrated it, it may imply that certain circadian rhythm could be induced; this would help them to adapt to the extra-uterine environment, so we carried out this consecutive study.

Subjects and Methods

Subjects

The study was approved by the hospital review board and informed consent was obtained from all parents following a detailed explanation of the study. Since it was a consecutive study, the recruitment was the same as before. The subjects in our former study[18] were recruited as the control group. In this study, we also chose ten premature neonates gestational age from 30 to 31 weeks. The work was performed as reported previously[18] except the babies were treated with NNS. Briefly, the recruiting standards were as follows. All infants were hospitalised 2-4 hours after their respective premature births. Infants with birth asphyxia, respiratory distress syndrome, apnoea, malformation, infection or haemolytic diseases were excluded from the study. The reasons for premature birth were twin pregnancy, bicornate uterus, placenta praevia and mother was fatigue. The infants were cared for in incubators with room lights. The environmental factors (esp. the lighting) were almost the same. Because the light was the very important entrainment to SCN, we were very cautious to keep it same. On the first day of their hospitalisation, they were fasting and on fluid replacement. On the second day, they were fed by nasal-gastric tubes (intermittent gavage) with formula for preterm infants every three hours, in addition to a regimen of intravenous nutrition as controls. Feeding regime and parental nutrition for supplement in both groups were as follows: All infants in both groups were hospitalised 2-4 hours after their respective premature births. On the first day of their hospitalisation, they were fasting and on fluid maintenance. On the second day, they were fed by nasal-gastric tubes with formula for preterm infants every three hours, in addition to a regimen of intravenous nutrition. The amount of the milk volume was started at 10 to 20 mL/kg/day and advanced by no more than 20 mL/kg/day. We started lipids

at 1.0 g/kg/day and advance by 1.0 g/kg/day to a usual maximum of 3 g/kg/day while monitoring and maintaining serum triglycerides at less than 200 mg/dL. We started amino acids at 1.5 to 2.0 g/kg/day and advanced by 1.0 g/kg/day to a usual maximum of 3.0 to 3.5 g/kg/day. Parenteral nutrition was continued until enteral feedings were established well and provided approximately 100 to 110 kcal/kg/day. Antibiotics were administered for 5 days before the blood culture indicated that there were no bacteria growing. They were not on oxygen and the vital signs were monitored throughout the study. Vital signs remained within normal limits, and SaO_2 was >85%. The results of routine laboratory examinations were normal. Total serum bilirubin was detected by a transcutaneous bilirubinometre (JM-103 Minolta Airshields).[19] If the data were over the cut-off value, the babies were placed on phototherapy.

NNS

In this study, all infants were provided NNS for 15 minutes every three hours before a tube feed for two weeks. First, the lower lip was gently stroked with a silica gel nipple, which was then moved intraorally to stimulate the tongue in a gentle front and back movement until sucking was induced. This nipple was kept in the mouth for NNS for 15 minutes.

Methods

Methods were the same as reported previously.[18] Briefly they are as follows:

Sample Acquisition

At 7am and 7pm on the 1st day, 7th day and 14th day of hospitalisation, 1.0 ml peripheral venous blood was obtained from the neonates and collected in a sterile tube with EDTA-K3 anticoagulant. The blood was immediately processed by centrifugation. PBMC were isolated using the Ficoll-Paque density gradient centrifugation method, then washed and frozen at -80°C until assay.

Total RNA Isolation and Reverse Transcription

Total cellular RNA was isolated from PBMC samples using TRIzol reagent according to manufacturer's description (Gibco BRL). RNA concentration was determined by spectrophotometry. RT reactions were carried out for each RNA sample in MicroAmp reaction tubes using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol.

Plasmid Construction

Plasmid was used to construct standard curves. First based on human homologues of the circadian genes in GeneBank, primers were designed which covered two or three introns of the respective genes. The sequences were as follows: *BMAL1:* forward (f) CTCCAGCCCATTAACATC, reverse (r) GCTACCAATGATGCTTCTGT, product size 638 bp. *CRY1:* f-TCCCAGGTTGTAGCAGCAGT, r-ATTTGGATTACGCACATTAT, 670 bp. *GAPDH:* f-GAAGGTGAAGGTCGGAGTC, r-GAAGAT GGTGATGGGATTTC, 225 bp. Then we performed PCR in a 50 µl reaction mixture containing 2 µl of cDNA, 1 µl of 0.2 µM of each primer, 1 µl of 10 mM dNTP, 5 µl of 10×PCR buffer, 4 µl of 25 mM MgCl2, 2.5 U of Taq polymerase. Each PCR reaction consisted of one cycle at 94°C for 5 minutes, followed by 40 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with an additional cycle of 72°C for 7 minutes at the end of the reaction. Products were purified and retrieved, then cloned.

Real Time PCR

Based on human homologues of the circadian genes in GeneBank, primers and probes were designed. The primers covered two introns of the purpose genes and the fragments were contained in the plasmid. The sequences were as follows:
BMAL1: f-CTCCAGCCCATTAACATC, r-GCCTCATCATTACTGGGACT, probe, 5'FAM-CTCCCCCTGATGCCTCTTCTCC-TAMRA- 3', product size 240 bp. *CRY1*: f-GCAGTGGAAGTTGCTCTCAAG, r-CTGAATGTTTTCTAATTAGTGC, probe, 5'FAM-AGGTGAG TTTGCTGACTGTCGCCAT-TAMRA-3', 196 bp. *GAPDH*: f-GAAGGTGAAGGTCGGAGTC, r-GAAG ATGGTGATGGGATTTC, probe, 5'FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA-3', 225 bp. Then we performed PCR in a 50 µl reaction mixture containing 2 µl of cDNA, 1 µl of 0.2 µM of each primer, 1 µl of 10 mM dNTP, 5 µl of 10×PCR buffer, 7 µl of 25 mM MgC l2, 2.5 U of Taq polymerase. Each PCR reaction consisted of one cycle at 94°C for 5 minutes, followed by 40 cycles at 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 40 seconds using a GeneAmp 5700 Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. Water controls were included to ensure specificity. The abundance of circadian genes mRNA expressed in molecules was normalised to the

abundance of *GAPDH* mRNA to control for sample-to-sample variation in total RNA input and RT efficiency.

Statistical Analysis

Patient characteristic values were expressed as mean±SD. Patient characters and gene expressions of two groups on the same time point were analysed with *t* test. χ^2 test was used for categorical variables. Same gene expressions in one group in different time points were analysed with ANOVA. Statistical significance was defined as p<0.05. SPSS 18 software was used for statistical analysis.

Result

Patient Characteristics

Patient characteristics were summarised in Table 1. In NNS group, seven babies were female and four babies were delivered by Cesarean Section. In control group, six babies were female and four babies were delivered by Cesarean Section. There were no statistical differences in terms of mean gestational age, mean admission age, mean birth weight and mean phototherapy time per capita between the two groups. Every baby needed phototherapy in both groups. Time to achieve full feeding was 11.4±1.7 day in NNS group, 13.8±3.1 day in control group, *t*=2.11, *p*=0.049.

Circadian Gene mRNA Expressions

Circadian gene mRNA values were normalised to the amount of *GAPDH* mRNA in each sample. In this study, in NNS group, the expression of *BMAL1* did not have a statistical difference between 7am and 7pm on the 1st day (Z=0.459, *p*=0.646), 7th day (Z=0.255, *p*=0.799) or 14th day (Z=0.153, *p*=0.878). The expression of *CRY1* did not have a statistical difference between 7am and 7pm on the 1st day (Z=1.122, *p*=0.262), 7th day (Z=0.408, *p*=0.683) or 14th day (Z=0.918, *p*=0.359).

In control group, the expression of *BMAL1* had no statistical differences, neither did the *CRY1* (for details, see reference 18).

At the same time point, the mean values of same gene expression of two groups were compared and there was no difference, see Table 2.

The gene expression fluctuation was in Figure 1. There is no obvious trend in the expressions of these two genes in either group. In group of NNS, *BMAL1*: F=0.181, *p*=0.969; *CRY1*: F=0.828, *p*=0.535. In control group, *BMAL1*: F=0.521, *p*=0.76; *CRY1*: F=0.549, *p*=0.74.

Table 1   Patient characteristics

	NNS	Control	*p value*
Male/female	3/7	4/6	0.639
C section/vaginal	4/6	4/6	1.000
Gestational age (week)	30.3±0.5	30.1±0.32	0.288
Admission age (hour)	3.35±0.71	3.1±0.91	0.501
Birth weight (gram)	1235±109	1257±126	0.683
Phototherapy time (hour)	19.2±4.7	18.4±4.5	0.703
Time to achieve full feeding (day)	11.4±1.7	13.8±3.1	0.049

Table 2   *BMAL1* and *CRY1* expressions in PBMC of two groups in the same time point

		BMAL1 expression*			*CRY1* expression*		
		NNS	Control	p	NNS	Control	p
7am	1st day	0.508±0.30	0.566±0.35	0.696	0.511±0.32	0.700±0.53	0.348
	7th day	0.475±0.23	0.517±0.22	0.681	0.754±0.37	0.663±0.40	0.602
	14th day	0.544±0.28	0.487±0.27	0.648	0.671±0.27	0.552±0.44	0.477
7pm	1st day	0.482±0.26	0.400±0.25	0.492	0.624±0.27	0.476±0.28	0.243
	7th day	0.483±0.25	0.514±0.32	0.787	0.621±0.30	0.685±0.42	0.703
	14th day	0.556±0.29	0.577±0.23	0.859	0.549±0.26	0.515±0.34	0.803

*mRNA values were normalised to the amount of GAPDH mRNA in each sample, and these values were the mean±SD.

Discussion

Our results suggest that preterm infants treated with NNS have no rhythm fluctuations in the circadian expression of *BMAL1* and *CRY1* in PBMC, the same as in non-NNS treated preterm infants.

Mice has a food-entrainable pacemaker outside the SCN, in which canonical clock genes such as *CRY1, CRY2* and *BMAL1* play essential roles in regulating FAA in a circadian oscillatory manner.[7] Jang et al[20] found that in peripheral tissue, such as liver, feeding period restriction altered the expression of peripheral circadian rhythm genes such as *BMAL1*, *CLOCK* and *PER*. The results of these two studies suggested that canonical clock genes play a role in the food-entrainable circadian rhythm.

During the first two weeks, preterm babies of gestational age between 30 to 31 weeks were unable to ingest adequate formula from a bottle; therefore, they were fed by using a nasal tube. Treatment with NNS prior to tube feeding made it easier for these infants to tolerate feeding. In fact, NNS could shorten the period of transition to full oral feeding, lessen the length of hospital stay, alleviate pain, improve breastfeeding rates among preterm infants, increase gastric motility, help insulin release into the gastric lumen in preterm infants, and decrease the risk of sudden infant death syndrome.

In our study, preterm babies provided with NNS did not show a fluctuation in *BMAL1* and *CRY1* circadian expression in PBMC. A possible cause could be that NNS is not real milk sucking, although it has many advantages. Nutritive sucking (NS) occurred in the presence of oral fluid whereas NNS took place in the absence of fluid. The rate of sucking was higher and the pressure amplitude of sucking was smaller during NNS than in NS.[21] The intensity of the stimulus to the circadian rhythm centre may be lower in NNS treatment than in NS. Lingual patterns during NS resulted in significantly greater displacements and excursions than NNS in both anterior and posterior regions of the tongue. In addition, the angle of hyoid movement during NNS was significantly smaller than the angle recorded during NS tasks.[22] The results of Lappi et al[23] demonstrated that NNS had no significant effect on heart rate or heart rate variability. Morren et al[24] found that NNS had no effect on cerebral or peripheral oxygenation. Due to these differences in dynamics between NNS and NS, NNS is not a real food entraining clue to the circadian rhythm centre.

A recent report suggested that daily rhythm of food-anticipatory behavioral activity in mice did not require the known circadian clock. Mutant mice lacking known circadian clock functions in all tissues exhibit normal food-anticipatory behavior both in a light-dark cycle and in constant darkness, regardless of whether the mutation disables the positive or negative limb of the clock feedback mechanism.[25] This result was contradictory to the results of Takasu et al[7] and Jang et al[20] If food entrainment of the circadian rhythm centre has no effect on either the positive or the negative limb of the clock feedback mechanism, NNS might not have any effect on the expression of *BMAL1* and *CRY1* in PBMC in preterm neonates. However, this requires further study.
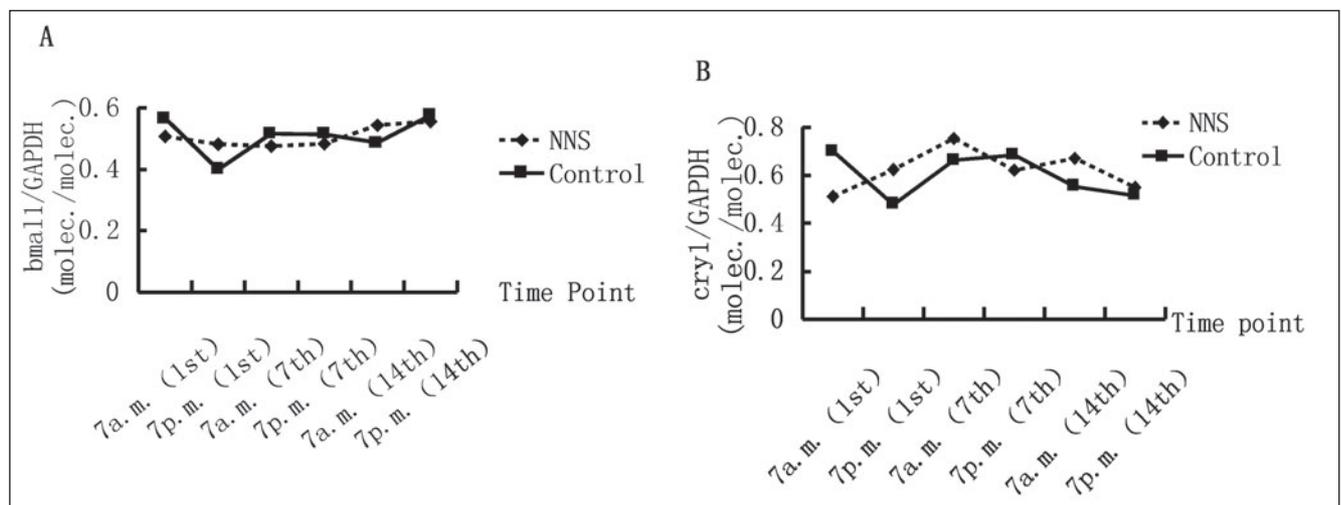

Figure 1   Fluctuation of two gene expressions. (A) fluctuation of *BMAL1*; (B) fluctuation of *CRY1*. 1st means 1st day of hospitalisation, 7th means 7th day of hospitalisation, 14th means 14th day of hospitalisation.

Our results suggested no obvious trends in the expression of these two genes. These infants were still very young; their gestational ages were between 30 to 31 weeks. We measured the gene expression during the first two weeks, which was the most unstable period of their life. A lack of adult-type salivary cortisol circadian rhythm was found in hospitalised preterm infants.[26]

Numata et al[27] evaluated the influence of circadian changes on swallowing and respiratory sinus arrhythmia (RSA) and found that the RSA amplitude during respiration with swallowing was larger in the morning than in the evening. Though there were no further studies on whether swallowing had an influence on circadian genes in PBMC, a nasal-gastric tube served as a constant stimulus to the esophagus and the swallowing mechanism which might negate the effect of NNS.

Kuroda et al[28] found that in mice the phase of the peripheral clocks was altered by the amount of food and the interval between feeding time points but was unaffected by the frequency of feeding, as long as the intervals remained fixed. In our study, although the time taken to achieve full feeding was shorter in NNS group than in the control group, according to our feeding regime and parental nutrition for supplement, the food amount was almost the same and the interval between feeding time points was fixed. Therefore, there was no influence of food amount and the intervals between feeding time on the fluctuation of *BMAL1* and *CRY1* circadian expression in PBMC.

One of the limitations of this study was that in our neonatal nursery, the room lights were always on. Although the light intensity was considered to be dim (<85 lux) and the exposure surface was limited, it was possible that the room lights may have affected the circadian rhythm and circadian gene expressions. Sometimes the infants required phototherapy to treat hyperbilirubinemia. These were all light cues that would be entrained to the SCN, and may have had some influence on the expression of *BMAL1* and *CRY1*.

Another limitation was that we could not continuously record the circadian genes expressions in these preterm neonates because of the ethical problems of repeated blood sampling.

Conclusions

The results of this pilot study suggest that NNS does not affect *BMAL1* and *CRY1* gene expression in PBMC in preterm neonates; further studies are wanted to confirm our findings.

Declaration of Conflicts of Interest

This paper has no financial support.

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