UNIVERSITY OF AUCKLAND

EFFECT OF CHRONIC INFLAMMATION ON CIRCADIAN RHYTHMS IN THE PRETERM FETUS

FMHS

SCHOLARSHIP IMPACT REPORT

MARCH 3RD 2017
Acknowledgements

I am grateful to my supervisor Professor Laura Bennet for her mentoring and induction to research in her lab, and to the University of Auckland for the support of this significant scholarship. I also thank the involvement of the preclinical subjects who produced the data I analysed, and the Health Research Council of New Zealand who generously funded the acquisition of the data.

Summary: Fetuses have rhythmic oscillations of physiological variables over a 24-hour period. These oscillations are founded by the mother: her own physiological variables also oscillate over a 24-hour period, and their variation entrains the fetus. In adults, these circadian rhythms – so called because they are circa diem: “about” a “day” – function to co-ordinate physiology. They help us to anticipate regularly occurring events, such as feeding, and also separate physiologically opposing behaviours, such as wakefulness and sleepiness. While we know that babies who are about to be born have circadian rhythms, there is less evidence that younger, preterm fetuses have these patterns.

In adult inflammation, circadian rhythms are disrupted. This allows us to mount a strong immune response, but at the cost of losing our daily rhythms. Usually this is an acceptable trade-off. However, in cases of non-resolving inflammation, the trade-off is one-sided: daily rhythms are lost, and the immune response is ongoing, with negative consequences. In pregnancy, fetal cardiovascular and body movement rhythms (commonly used in obstetrics as markers of fetal health) do not appear affected by inflammation. However, some aspects of neurological patterns are disturbed. Inflammation during gestation can lead to poor neurodevelopment and brain health in adult life; crucially, common obstetric measures of fetal health are inadequate to determine whether the fetus is in neurological distress.

This scholarship has been a rewarding opportunity to take my modest background in adult chronobiology and expand laterally into a relatively young field. Shifting into a comparatively unexplored area was a totally new experience. I have had difficulty acquainting myself with the literature; obviously, fetal physiology is very different to that of the adult. I was also moving from a clinical and molecular-medicine focused approach to a preclinical and integrative-physiology perspective, which has been challenging, in a good way! The ability to compile and assimilate the literature in a meaningful way was a steep learning curve (which I am still on!) but I am grateful for the chance to ground myself before I begin a project.

Input

My summer scholarship provided a stipend for me to undertake this project. The summer studentship fund did not support the project. The data analysed in this study came from studies supported by the Health Research Council of New Zealand (12/613).
Methods (Activity)

**Rationale:** Rhythmicity in physiology is a part of healthy living, allowing us to coordinate our physiology in anticipation of events and also separate out behaviours with opposing effects (1). Rhythmicity is cued by the external environment and internal timekeepers (2). Disruption of rhythmicity increases lifetime disease risk. We see this in adults who are chronically disrupted by shift work (3). We also see this chronodisruption in adults who are in a state of non-resolving (chronic) inflammation, such as in cancer, diabetes or fatigue (4, 5). The effects are like jet-lag: there is a ‘phase shift’, where physiological events or behaviours which would normally occur at a certain time, occur earlier or later. In experimental bacterial infection, there is a ‘phase delay’, where the cued behaviours are delayed by up to six hours (6). Such a shift may seem insignificant, but it accounts for half an average light period, and over several days and across several behaviours, can become very disruptive. Essentially, there is a bidirectional relationship between the circadian system and the immune system (7).

The fetus also has rhythms, which are cued from its external environment: the mother (8). The point of these rhythms is not yet determined, but it is likely they exist for the same reasons as in the adult: co-ordination of physiology. The fetus has strong cardiovascular and neurophysiological rhythms: in the fetal brain, strong metabolic rhythmicity is present, indicating compartmentalization of energy for brain growth and development (8). If a phase shift occurs in the fetus, it would change the way the fetus responds to energy and energy use. Could this affect development?

Babies are susceptible to inflammation in the womb, for example, from infection. The maternal/external environment may be in a state of chronic inflammation, such as in diabetes (5). Furthermore, already vulnerable babies, such as preterms, are often inflamed through conditions such as chorioamnionitis (9). Any one of these circumstances can disrupt the rhythmicity cues the fetus receives (8). Inflammation is known to affect the development of the fetus and chronic inflammation in particular has long-reaching effects (10). The brain is an especially energy-hungry organ. Interruptions to the energy supply, such as during inflammation, can cause impaired brain development and injury (11, 12). We know that disrupted/dysregulated brain metabolism plays a large role in brain injury (11, 12). This scholarship investigated whether chronic inflammation affects the circadian biology of the fetus and whether this contributes to altered brain development.

**Aims:** To evaluate pre-clinical data of the effects of inflammation induced by low-dose lipopolysaccharide (LPS) on circadian rhythms of preterm fetuses.

**Hypothesis:** Chronic inflammation induced by LPS causes chronodisruption in fetal physiological behaviour; this results in a mismatch in energy supply and demand, which may impact development.
**Methods:** Experimental setup: Pregnant ewes were entrained to a 12:12 light/dark cycle (light period from 6am to 6pm) for two weeks in the laboratory environment. Fetal sheep at 0.7 gestation (equivalent to human gestation of 28-30 weeks) were instrumented with catheters and electrodes for measurement of fetal heart rate (FHR) mean arterial pressure (MAP), carotid blood flow (CaBF) as an index of cerebral perfusion, body movements (nuchal electromyographic (EMG) activity, and electroencephalographic (EEG) amplitude (log transformed to decibels) and EEG spectral edge (SE) frequency. Ewes and fetuses were given five days to recover from surgery, and then assigned to either saline infusion (n=8) or low-dose ramping LPS infusion[LB1] (n=8) for five days. The LPS delivery was doubled each day, from 200ng in the first day to 3200ng in the fifth day. Fetuses were studied for a further five days without infusion and then killed for tissue collection and post-mortem analysis.

Recording and analysis: The fetal physiological signals were averaged hourly from 24 hours before the start of saline or LPs infusion, until the end of the experiment. I ensured that the records for each fetus were synchronised for the time of day to ensure accuracy of circadian pattern analysis. Data were statistically compared using repeated measures analysis of variance (SPSS, v22). Where statistical differences were found, post-hoc testing was undertaken using the least-square difference test. Statistical significance was accepted when P<0.05. Data are presented as mean±SEM.

**Results (Output)**

*Fetal heart rate:* A daily cycle in FHR (Fig. 1) was observed, with a peak in the early evening before the dark period, and a nadir in the morning at the beginning of the light period. There was no significant difference in the patterns observed between groups. There was a significantly higher heart rate during the first and third day.

![Figure 1](image)

**Figure 1** This figure shows the percentage changes in fetal heart rate (FHR, beats per minute) from 24 hours before either a 5 day saline (open symbols) or LPS infusion (closed symbols) until 5 days after infusion. * P<0.05. Data are 1hr mean±SEM.
*Fetal blood pressure:* The circadian pattern in fetal MAP was more subtle, with a rise in pressure during the day, and plateau or dip at night (Fig 2).

**Figure 2** This figure demonstrates the changes in mean arterial pressure (MAP, mmHg) over four day-night cycles. The arrows in the last three night periods indicate where nightly ‘dipping’ may be observable. This pattern was more strongly observed in the LPS group, with an attenuation of the pattern in the vehicle group seen during the saline infusion. FHR was lower in the LPS group during day 3 or LPS infusion (P<0.05), but overall there was no differences between groups for basal value or circadian pattern (Fig 3).

**Figure 3** This figure shows the changes in mean arterial pressure (MAP, mmHg) before, during and after saline (open circles) or LPS (close circles) infusion. *P<0.05. Data are 1hr mean±SEM*
Carotid blood flow: There was no clear circadian oscillation in CaBF (Fig 4). There was, however, a progressively greater rise in CaBF over the course of the experiment, with CaBF significantly higher than control group values at the end of the infusion period and during the last three days (P<0.05).

**Figure 4** This figure shows changes in carotid blood flow (CaBF, mL/min) before, during and after saline (open circles) or LPS (close circles) infusion. *P<0.05. Data are 1hr mean±SEM.

Fetal body movements: There was a clear circadian pattern in nuchal EMG activity, until the last three days of the experiment (Fig 5). During this time, the pattern became noticeably attenuated. There was no significant difference between groups.

**Figure 5** This figure shows changes in nuchal EMG activity before, during and after saline (open circles) or LPS (close circles) infusion. *P<0.05. Data are 1hr mean±SEM.
**EEG activity:** In the control group, EEG power increases with fetal age. A subtle diurnal cycle of increasing power was seen in the control fetus, with greater power increases observed during the day, and a plateau overnight. LPS administration was associated with a trend for EEG suppression, but this was not significant.

![EEG Power Chart](chart1.png)

**Figure 6** This figure shows changes in electroencephalographic (EEG) activity before, during and after saline (open circles) or LPS (close circles) infusion. Data are 1hr mean±SEM.

**EEG – spectral edge frequency:** A subtle circadian pattern was observed in SE, with activity reciprocal to EEG power (Fig 7). LPS was associated with a significant reduction in SE frequency after LPS infusion, until the last day.

![SE Chart](chart2.png)

**Figure 7** This figure shows changes in EEG in spectral edge frequency activity activity before, during and after saline (open circles) or LPS (close circles) infusion. *P<0.05. Data are 1hr mean±SEM.
Discussion (Outcomes)

The overarching objective of this study was to examine whether the preterm fetus has circadian rhythms, and whether fetal systemic inflammation induced by exposure to the endotoxin LPS would change these patterns. I used a preclinical animal model, the fetal sheep, to examine this. My data shows that the preterm sheep fetus has circadian rhythms in body movements, and cardiovascular and neurophysiological parameters. The strongest rhythms were seen in heart rate and body movements, whereas the patterns were more subtle in brain activity and in blood pressure and blood flow. Inflammation did not appear to have a significant effect on circadian patterns. There were, however, effects of inflammation on the maturation of blood flow and brain activity. I discuss some of my key findings below.

The fetal gym: All fetuses are active at any age. They are constantly exercising to increase muscle strength and in terms of the brain, promoting brain development and connections. However, the preterm fetus is a very active creature compared to its older brethren. The preterm fetus is constantly moving and making breathing-like movements (for lung development), licking, swallowing, and making eye movements (13). The fetal EEG is “discontinuous”, that is, the EEG is comprised of mixed EEG amplitudes and frequencies, without apparent patterns (14). This is in contrast to term, where the fetus compartmentalises energy by linking behaviour to brain activity (14). The more mature fetus starts to develop EEG activity states which are analogous to sleep states such as rapid eye movement (REM) sleep and non-REM sleep (14). During REM the fetus makes eye movements and breathing movements and licks and swallows, while in non-REM sleep the fetus suspends these activities in favour of body movements (15). We have similar behaviour as adults, where we are essentially paralysed in REM sleep, and active in non-REM. The only difference is we don’t switch off our breathing; this is unique to the fetus who has the placenta to do the work of our lungs in providing oxygen.

Why compartmentalise? The older fetus does this because it cannot obtain enough energy to support simultaneous activities and still grow. This is not a problem for the smaller, more hypometabolic fetus. This lower metabolic demand and apparent chaotic continuous behaviour is one of the reasons why a circadian pattern was not thought to be present in the preterm fetus. However, my data show that the preterm fetus does have a circadian pattern and that the preterm fetus expresses different behaviours at slightly different times. Importantly, they fit the cycle of maternal energy use and rest.

Heart rate and the late afternoon: The clearest circadian pattern is in fetal heart rate, where there is typically an increase in activity towards late afternoon and early evening. This pattern is also seen in adults (16), and the heart is said to have an internal circadian clock (17). Maternal cues may influence activation of the fetal heart clock, synchronising its activity during this time. Increased activity on a regular basis is important for heart function. The clarity of the circadian pattern may relate to heart rate.
being set by the pacemaker – a set basal beat which can be increased and decreased by changes in autonomic function. Fluctuating signals such as blood pressure and brain activity are less clear. In this respect, body movements display a clearer diurnal rhythm, suggesting that their activity has a relatively set pacemaker. Future work will evaluate fetal heart rate variability to understand the nature of the cardiac circadian changes. This, and fetal heart rate, are gold standard measures of fetal well-being in obstetrics.

The fetal party animal at night: While most increased heart activity clustered in the late afternoon and early evening, the body movements and brain activity of the fetus is most active at night. The team has previously shown, using near infrared spectroscopy, that fetal mitochondrial ATP production is also increased at night (18). That the fetus is energetic at night fits with the activity reduction in maternal phase of rest (even sheep are generally at rest at night). The fetus can utilise energy from the last meal to increase activity and my data would suggest that night time is important for fetal brain development. I could not see an association with blood flow to the brain, but this may reflect the use of a surrogate measure.

Curiously, there is a blunting of the nuchal activity during the late stages of recording. It is unclear what is happening here, and does not relate to LPS treatment. However, it is occurring during a gestational time when the fetus is transitioning from the simultaneous behaviour/discontinuous EEG to the more mature patterns of behaviour and sleep state cycling; there may be some change in regulatory control of activity and energy utilisation.

Day time is grow time: It is difficult to see this trend, but a snapshot over a few days reveals that fetal blood pressure rises during the day and plateaus (and sometimes dips) at night. The rise in fetal blood pressure reflects increased vascular resistance which in turn correlates with fetal growth. My data thus suggests fetuses may grow largely during the day, which would be consistent with the maximum nutrition available to mum. This is a completely novel finding. Further, my data suggest that the fetus may experience nocturnal dipping. Blood pressure ‘dipping’ at night is an established pattern in healthy adults and autonomic function (19) but has not been reported in the fetus to date.

I observed there was a loss of rhythm during the infusion phase, particularly in the control group, and a few days of pressure plateau. This potentially reflects a sensitivity of the fetus to experimental procedures – blood sampling and infusion. In future experiments this will need to be examined further to see if we can reduce the impact on the fetus of what we do as scientists.

The consequences of inflammation: On the positive side, chronic inflammation does not appear to markedly change fetal circadian patterns, although more detailed analysis needs to be performed. However, inflammation clearly had an impact on brain development, with EEG amplitude and frequency lower than controls. Frequency was particularly affected, and this would reflect reduced development of the neural network.
This finding is consistent with the clinical observation that inflammation is associated with impaired maturation of white matter cells in the brain such as oligodendrocytes, which myelinate neurons, and impaired cortical connectivity (10). Further analysis of EEG activity, including fast Fourier analysis of frequency, will tell us more about how the brain is developing, along with histopathological assessment of the brain.

It is notable that LPS was associated with vasodilatation, meaning reduced EEG activity was not due to hypoperfusion of the brain (coupling of blood flow and metabolism). LPS is associated with both altered sensitivity to vasodilators and vasoconstrictors (20).

Conclusions: The preterm fetal sheep has established circadian patterns which associate with maternal rest and energy utilisation phases. The fetus appears to grow during the day when energy supply is maximal, and use the evening and night for exercise which require less energy. However, my hypothesis that LPS would cause the fetus to become chronodisrupted was not supported by the data.

Perspectives (Impact)

A womb with a view: My study has provided a fascinating insight into life before birth and how the fetus can experience the outside world through the biological cues provided by mum. Our world is regulated by the rising and setting of the sun, which in turn dictates when we are active and when we consume energy. This is the role circadian patterns play in regulating our biological processes.

My study demonstrates that the fetal world is also dependent on these cues; or more accurately, upon mum’s adherence to these routines and well established circadian patterns so that she may pass the cues on in a timely manner. This gives the fetus the energy to grow and to develop at the right times with the right energy sources. In today’s world, however, these critically important patterns are under assault. How many of us eat on schedule and get enough sleep, how many of go to bed with our cell phones or iPads with their glowing screens? Chronodisruption is now recognised to contribute to increased risk of illness and disease, and my data show the dependence of normal fetal growth on circadian timing: they are also at risk of chronodisruption.

Limited clinical data suggest that chronodisruption in pregnant shift workers is associated with poor fetal growth (21). My data support the need for Kiwi mums to be advised about the importance of maintaining eating and sleeping habits (sleep hygiene) during pregnancy for their health and the health of their babies.

My study showed that low-grade fetal inflammation does not cause chronodisruption. Whether a more marked maternal inflammation would cause this is not known, but conditions associated with inflammation such as obesity, diet and diabetes are also associated with poor fetal outcomes. The lack of effect is, however, not a cause for immediate celebration as my data clearly show that exposure to LPS is associated with altered brain maturation, as determined by the functional measure of EEG consistent
with the clinical data which shows that inflammation has catastrophic effects on neurodevelopment, and long-lasting consequences for behaviour and health in adult life (10). There is also interest in using biomarkers as indicators of fetal health in the womb and as predictors of fetal distress. Two of the gold standard measures used in obstetrics are fetal heart rate and body movements (15). The preliminary data analysed in this scholarship suggests that these measurements, on their own, are insufficient to determine whether the fetus is experiencing impaired neurodevelopment.

FOR MORE INFORMATION PLEASE CONTACT:

Laura Bennet | Professor
Physiology | The University of Auckland
T DDI : +64 9 373 7599 | Ext: 84890
E l.bennet@auckland.ac.nz

Faculty of Medical and Health Sciences
85 Park Road
Grafton
Auckland 1023
References


