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Early sinusoidal heart rate patterns and heart rate variability to assess hypoxia-ischaemia in near-term fetal sheep

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Key points

- Therapeutic hypothermia needs to be started as early as possible in the first 6 hours after acute hypoxic-ischaemic (HI) injury, but the severity and timing of HI are often unclear. In this study we evaluated whether measures of heart rate variability (HRV) might provide early biomarkers of HI.
- The duration but not magnitude of suppression of HRV power and conversely, increased sample entropy of the heart rate were associated with severity of HI, such that changes in the first 3 hours did not discriminate between groups.
- Relative changes in HRV power bands showed different patterns between groups and therefore may have potential to evaluate the severity of HI.
- Aberrant fetal heart rate patterns and increased arginine vasopressin levels in the first hour after moderate and severe HI were correlated with loss of EEG power after 3 days recovery, suggesting potential utility as early biomarkers of outcome.

Abstract

Therapeutic hypothermia is partially neuroprotective after acute hypoxic-ischaemic (HI) injury, likely because the timing and severity of HI are often unclear, making timely recruitment for treatment challenging. We evaluated the utility of changes in heart rate variability (HRV) after HI as biomarkers of the timing and severity of acute HI. Chronically instrumented fetal sheep at 0.85 gestational age were exposed to different durations of umbilical cord occlusion to produce mild (n=6), moderate (n=8) or severe HI (n=8) or to sham occlusion (n=5). Heart rate (HR) and HRV indices were assessed until 72 hours after HI.

All HI groups showed suppressed very-low frequency HRV power and elevated sample entropy for the first 3 hours; more prolonged changes were associated with greater severity of HI. Analysis of relative changes in spectral power showed that the moderate and severe groups showed a shift towards higher HRV frequencies, which was most marked after severe HI. This shift was associated with abnormal rhythmic HR patterns including sinusoidal patterns in the first hour after HI, and with elevated plasma levels of arginine vasopressin, which were correlated with subsequent loss of EEG power by day 3. In conclusion, absolute changes in HRV power in the first 3 hours after acute HI were not significantly related to the severity of HI. The intriguing relative shift in spectral power towards higher frequencies likely reflects greater autonomic dysfunction after severe HI. However, sinusoidal HR patterns and elevated vasopressin levels may have utility as biomarkers of severe HI.

Michi Kasai is an obstetrician at Yokohama City University School of Medicine, Japan. Clinical questions led her to study fetal physiology with professors Laura Bennet and Alistair Gunn. She aims to understand fetal physiologic response to hypoxic events, especially changes in fetal heart rate, to identify the timing of medical intervention for better fetal outcome. **Christopher Lear** is a Research Fellow with the Fetal Physiology and Neuroscience Group, University of Auckland, New Zealand. His interests include the physiological adaptation to labour and how understanding this fundamental physiology can improve the identification of fetuses at risk of hypoxic-ischaemic brain injury.

Introduction

Hypoxia-ischaemia (HI) around the time of birth remains a significant cause of neurodevelopmental disability (Lee *et al.*, 2013). Therapeutic hypothermia is now well established to partially improve outcomes after moderate-severe HI encephalopathy (HIE) in term and near-term infants. To be most effective, hypothermia needs to be initiated as soon as possible during the latent phase after HI, typically the first 6 hours after HI, before the onset of secondary neural deterioration as shown by delayed onset of seizures and evolving cell death (Wassink *et al.*, 2018). Delay within the latent phase reduces the effectiveness of treatment (Sabir *et al.*, 2012; Wassink *et al.*, 2018). Moreover, in some cases it is likely that HI was initiated well before birth, further reducing the time for intervention. Thus, it remains challenging to identify infants early in the latent phase of injury, and accurately assess the severity of HI (Bennet *et al.*, 2010).

Heart rate variability (HRV) is under autonomic neural control (Akselrod *et al.*, 1981; Koome *et al.*, 2014; Lear *et al.*, 2016), and so changes in HRV may provide information on the timing and severity of HI injury. Clinically, suppression of HRV from 6 hours after birth and later amongst infants undergoing hypothermia is correlated with EEG abnormalities, the severity of MRI-based brain injury and long-term neurodevelopmental outcome (Massaro *et al.*, 2014; Goulding *et al.*, 2015; Metzler *et al.*, 2017). In preterm fetal sheep, severe HI is associated with initial suppression of HRV in the early latent phase, which returned to 'normal' levels of HRV from 2 hours after HI (George *et al.*, 2004; Yamaguchi *et al.*, 2018). This initial recovery was followed by delayed suppression of HRV after the secondary deterioration. However, the relationship between the severity of HI at term equivalent and recovery of heart rate (HR) and HRV in the critical latent phase is unclear. Sinusoidal fetal heart rate patterns have been reported after asphyxia in fetal sheep, and can be induced by combined parasympathetic-blockade and arginine vasopressin (AVP) infusions (Murata *et al.*, 1985), suggesting the hypothesis that the presence of sinusoidal fetal HR (FHR) patterns or elevated levels of AVP after HI may be useful biomarkers of asphyxia.

Thus, in the present study we evaluated in near-term fetal sheep the relationship between suppression of fetal HRV or the presence of sinusoidal FHR patterns or elevated AVP levels and mild, moderate and severe HI induced by complete umbilical cord occlusion (UCO) for different durations. We then assessed whether the presence of sinusoidal FHR patterns or elevated AVP levels after UCO were predictive of recovery of EEG power after 3 days recovery.

Materials and Methods

Ethical approval

All procedures were approved by the Animal Ethics Committee of the University of Auckland following the New Zealand Animal Welfare Act 1999, and the Code of Ethical Conduct for animals in research established by the Ministry of Primary Industries, Government of New Zealand. All procedures comply with the guidelines of the Journal of Physiology (Grundy, 2015).

Surgical procedures

27 Romney/Suffolk fetal sheep were operated on at 117–125 d gestational age (term=147 days) (Hunter *et al.*, 2003; Drury *et al.*, 2014). Food, but not water was withdrawn 18 hours before surgery. Ewes were given 5 ml of Streptocin (procaine penicillin (250,000 IU/ml) and dihydrostreptomycin (250 mg/ml), Stockguard Labs, Hamilton, New Zealand) intramuscularly 30 min before the start of surgery. Anaesthesia was induced by intravenous injection of propofol (5 mg/kg; AstraZeneca, Auckland, New Zealand), and general anaesthesia maintained using 2–3% isoflurane (Medsource, Ashburton, New Zealand) in oxygen. All surgical procedures were performed using sterile techniques. Catheters were placed in the left fetal femoral artery and vein, right brachial artery and vein, and the amniotic sac. Two pairs of EEG electrodes (Cooner Wire, Chatsworth, CA, USA) were placed through burr holes on the dura over the parasagittal parietal cortex (10 mm and 20 mm anterior to bregma and 10 mm lateral) and secured with cyanoacrylate glue. A reference electrode was sewn over the occiput. A pair of electrodes was sewn over the fetal chest to measure the fetal electrocardiogram (ECG). Another pair of electrodes was sewn into nuchal muscle to measure nuchal electromyographic (EMG) activity. An 18–20 mm diameter inflatable silicone occluder was placed around the umbilical cord (In Vivo Metric, Healdsburg, CA, USA). All fetal leads were exteriorised through the maternal flank and a maternal long saphenous vein was catheterised to provide access for postoperative care and euthanasia. 80 mg gentamicin (Rousell, Auckland, New Zealand) was administered into the amniotic sac before closure of the uterus.

Post-operative care

All sheep were housed in separate metabolic cages with access to water and food ad libitum, together in a temperature-controlled room (16 ± 1°C, humidity 50 ± 10%) with a 12-hour light/dark cycle. Four days post-operative recovery was allowed before experiments, during which time antibiotics were intravenously administered to the ewe daily (600 mg benzylpenicillin sodium;

Novartis, Auckland, New Zealand, and 80 mg gentamicin). Fetal catheters were maintained by continuous infusion of heparinized saline (20 U/ml at 0.2 ml/h).

Experimental Recordings

Fetal mean arterial blood pressure (MAP), corrected for maternal movement by subtraction of amniotic fluid pressure (Novatrans II, MX860; Medex Inc., Hilliard, OH, USA), ECG, EEG and EMG were recorded continuously from 24 hours before until 72 hours after UCO. The blood pressure signal was collected at 64 Hz and low pass filtered at 30 Hz. The raw ECG signal was analogue filtered with a first-order high- pass filter set at 1 Hz and an eight-order low-pass Bessel filter set at 100 Hz and saved at 1024 Hz and used to derive fetal HR and fetal HRV, as described below. Data were collected by computer and stored to disk for offline analysis.

The nuchal EMG signal was band-pass filtered between 100 Hz and 1 kHz, the signal was then integrated using a time constant of 1 sec. The analogue fetal EEG signal was low pass filtered with the cut-off frequency set with the -3 dB point at 30 Hz, and digitised at 256 Hz (using analogue to digital cards, National Instruments Corp., Austin, TX, USA). EEG power was derived from the power spectrum signal between 0.5 and 20 Hz.

Experimental protocol

Experiments were performed at 121–129 d (0.85) gestation. At this age the neural maturation of the fetal sheep is broadly equivalent to term in human infants (Barlow, 1969). Fetuses were randomly assigned to UCO (n=22) or sham occlusion (n=5). UCO was induced between 0900 and 1000 h by rapid inflation of the umbilical cord occluder with sterile saline of a defined volume known to compress the umbilical cord. Successful occlusion was confirmed by observation of a rapid onset of bradycardia with a rise in MAP, and by pH and blood gas measurements. Fetuses were randomly assigned to three different durations of UCO: 10 min (n=6), 15 min (n=8), or >15 min until MAP reached 8 mmHg (n=8). These durations and depths of occlusion were determined in previous studies (Hunter *et al.*, 2003; Drury *et al.*, 2012; Drury *et al.*, 2013; Drury *et al.*, 2014) to reflect mild, moderate and severe HI. Occlusions were ended early if the fetus became asystolic, or in the 10 min and 15 min groups if MAP fell below 10 mmHg. After release of UCO, fetuses were allowed to auto-resuscitate. If fetal HR was not above 100 bpm within one minute of the end of occlusion; 0.1–0.3 ml/kg of 1/10000 adrenaline (Hospira, Auckland, New Zealand) was administered via the brachial vein. For sham occlusion, the occluder was not inflated.

Fetal arterial blood samples

Fetal arterial blood samples (0.3 mL) were taken before the start of UCO, and during UCO at either 8 min (mild HI) or 12 min (moderate and severe HI) after the start of UCO, and then 1, 2, 4, 6, 24, 48 and 72 hours after the end of UCO. Whole blood samples were analysed for fetal pH and blood gases (Ciba-Corning Diagnostics 845 blood gas analyser and co-oximeter, MA, USA) and for glucose and lactate measurements (YSI model 2300, Yellow Springs, OH, USA). 3 mL plasma samples were collected before and at 12 minutes during UCO, and at 1, 3, 6, 24, 48 and 72 hours after the end of UCO to measure AVP concentrations using extracted radioimmunoassay, as previously described (Sadler *et al.*, 1983). The assay has a mean detection limit of 0.3 pmol/L and a within assay coefficient of variation of 6% at 1.5pmol/L, 3.6% at 4pmol/L and 10.8% at 16pmol/L. Unfortunately, plasma samples were not available for the mild HI group and from one fetus in the severe HI group.

Data analysis

Off-line analysis of the physiological data was performed using customized LabVIEW-based programs (National Instruments). All measures were averaged in 10-minute epochs during the first 24 hours after HI and into 1-hour epochs during the baseline period and from 24 to 72 hours after HI. Fetal HRV analysis was performed using Kubios Standard HRV version 3.1.0 (Kubios, Kuopio, Finland). All measures were assessed on continuous, non-overlapping 5-minute epochs based on recommendation of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Individual epochs were then averaged in 10-minute intervals for the first 24 hours after HI and in 1-hour epochs for the baseline period and from 24-72 hours after HI. Frequency domain measures of fetal HRV were calculated by using power spectrum density estimate, using Fast Fourier transformation. The frequency band boundaries used in this study were: very-low-frequency (VLF), 0-0.04 Hz; low-frequency (LF), 0.04-0.15 Hz; and high-frequency (HF), 0.15-0.4 Hz (Koome *et al.*, 2014; Shaw *et al.*, 2018). The power of each frequency band was calculated as the natural logarithm of the amplitude. The relative power of each frequency bands was also calculated in proportion to the total power present between 0-0.4 Hz. The non-linear measure sample entropy (SampEn) was calculated as a measure of complexity of the fetal HR patterns using vector m=2 and tolerance r=0.2.

Additionally, one second means of fetal HR were calculated and visually inspected by an experienced obstetrician (MK) blinded to the groups. Rhythmic patterns were classified as either sinusoidal or sinusoidal-like patterns. The sinusoidal pattern was defined as a rhythmic, undulating pattern resembling a sine-wave with minimal distortion or additional features (Modanlou & Murata, 2004; American College of Obstetricians and Gynecologists, 2010). Sinusoidal-like patterns were classified as having a similar rhythmic nature, period and frequency to those classified as sinusoidal, but without a true sine-wave

appearance or with additional superimposed repetitive features. The duration of all rhythmic patterns lasting longer than three minutes within the first 2 hours after HI was calculated. The total duration of these patterns within the first 2 hours after HI were then correlated against AVP concentrations 1 hour after HI in the moderate and severe HI groups. The relationship between the duration of sinusoidal-like patterns and AVP levels and subsequent loss of EEG power at day 3 after HI were assessed by linear regression.

Statistical analysis

Data were analysed using SPSS version 25 (IBM, Armonk, NY, USA). Between groups comparisons of serial data were performed by two-way analysis of variance, with time treated as a repeated measure between sham group and each HI groups. When there was a significant difference in baseline values between groups, two-way analysis of variance was performed, with baseline values treated as a covariate. For FHR pattern analysis, between groups comparisons of serial data were performed by two-way analysis of variance, with time treated as a repeated measure. When statistical significance was found, post hoc analysis was undertaken using Fisher's protected least significant difference test. Linear regression analysis was performed to compare AVP levels with the duration of rhythmic and sinusoidal patterns within moderate and severe HI groups. Statistical significance was accepted when $P<0.05$.

Results

Baseline parameters

All fetuses were healthy before UCO based on our laboratory standards (judged by blood gases, glucose, lactate, FHR, MAP, EEG, and EMG). There were no significant differences between the groups in fetal sex or number of twins. The composition of each group was: controls (n=5, 1 male, 4 females, no twins), mild HI (n=6, 2 males, 4 females, 1 twins), moderate HI (n=8, 3 males, 5 females, 1 twins) and severe HI (n=8, 3 males, 5 females, 4 twins). There were no differences in the baseline physiological parameters between groups except for lower high frequency heart rate variability in the severe HI group ($P<0.05$ vs control).

Blood gas analysis

All HI groups developed profound mixed acidosis and hypoxaemia during UCO (Table 1). Most parameters resolved to sham levels within the first few hours after UCO. Severe HI was associated with prolonged elevation of BE and lactate (BE; $P<0.01$, lactate; $P<0.005$) and a secondary increase in glucose at 24 hours ($P<0.01$) and 48 hours ($P<0.05$) compared to the control group. Adrenaline was required after UCO in 2/8 fetuses in the moderate group and 6/8 in the severe group.

Changes in fetal heart rate and arterial pressure after HI

The early recovery from HI was associated with marked dynamic changes in FHR, which frequently crossed control values. Mild and moderate HI were associated with brief initial tachycardia followed by a relative fall in HR, which resolved to sham levels within 6 hours after HI. The average values were not significant (2 way ANOVA). The maximum FHR after HI was significantly greater after both mild and moderate HI compared to controls (mild; $P<0.0005$, moderate; $P<0.05$, data not shown). By contrast, severe HI was associated with immediate tachycardia followed by a rapid but transient fall in HR within the first hour after HI. Progressive tachycardia then developed until approximately 9 hours after HI (Figure 1). The maximum FHR in the latent and secondary phases respectively was significantly greater than in the control group (latent phase; $P<0.0005$, secondary phase; $P<0.05$, data not shown).

There was no significant change in mean MAP in the first 3 hours after HI, but the maximal increase from baseline after moderate or severe HI was significantly greater than in controls (moderate; $P<0.005$, severe; $P<0.0005$, data not shown). All HI groups showed significantly increased MAP compared to controls in the secondary phase ($P<0.05$; mild: 6-18 hours, moderate: 6-48 hours, severe: 12-48 hours).

EEG and nuchal EMG activity

EEG power was initially suppressed in all HI groups (P<0.001 vs controls) and partially improved over the first 6 hours after HI (Figure 2). Moderate but not mild HI showed ongoing reduced EEG power for the 72 hours of recovery (P<0.01 vs. controls), whereas severe HI was associated with suppression from 0-12 hours after HI (P<0.05) and from 24 hours until the end of experiment (P<0.01). Fetal nuchal EMG activity was decreased for 3 hours after mild and moderate HI (P<0.05 vs controls), whereas severe HI was associated with suppression for the first 6 hours (P<0.05). By contrast, moderate HI was associated with delayed suppression from 12-24 hours (P<0.05 vs controls).

Fetal heart rate variability in the latent phase (0-6 hours)

In the mild HI group, the absolute power of all three fetal HRV frequency bands was significantly suppressed for the first 3 hours after HI (VLF; P<0.01, LF; P<0.005, HF; P<0.01, vs. controls, Figure 3). This suppression was uniform across power bands as shown by the lack of significant change in relative powers during this time (Figure 4). Moderate and severe HI were associated with marked suppression of VLF power for the first 6 hours after HI (moderate; P<0.05, severe; P<0.01, vs. controls); severe HI was also associated with suppression of LF until 3 hours (P<0.05 vs. controls, Figure 3). Interestingly, whereas the severe group showed reduced relative VLF power for the first 6 hours (P<0.05), relative LF power was elevated (P<0.05) with a similar but N.S. trend for relative HF (Figure 4). SampEn was significantly increased after mild and moderate HI for 3 hours (P<0.05, vs. controls, Figure 4), while it was increased for the first 6 hours after severe HI (P<0.01).

Fetal heart rate variability in the secondary phase (6 –72 hours)

Mild and moderate HI were associated with control values of fetal HRV power throughout the secondary phase. Severe HI was associated with continued suppression of VLF power from 0-12 hours (P<0.05 vs controls), suppression of LF power from 6-12 hours and 18-24 hours (P<0.05 vs controls). There was a trend for secondary suppression of HF power from 24-48 hours (P=0.076 vs controls, Figure 3). This delayed loss of HRV power after severe HI disproportionately affected the LF frequency band as shown by a reduction in relative LF from 18-24 hours (P<0.01, Figure 4), and a corresponding significant increase in relative VLF power from 18-48 hours (P<0.05). Severe HI was also associated with a sustained increase in SampEn from 0-24 hours after HI (P<0.05, vs controls), followed by resolution to control values (Figure 4).

Fetal heart rate patterns in latent phase and AVP levels after moderate and severe HI

The first 1 to 2 hours after UCO, the moderate and severe HI groups showed irregular fetal HR patterns, including intermittent episodes of rhythmic patterns as well as episodes of visually increased variability. Among the rhythmic patterns were patterns that were consistent with the obstetric sinusoidal fetal HR pattern. Other FHR patterns were seen that had a similar rhythmic nature but showed more complex or distorted waveforms, which may be described as sinusoidal-like patterns (see examples in Figure 5). These patterns were seen almost entirely in the early latent phase, within two hours of the end of UCO. Only severe HI was associated with an overall significant increase in the combined frequency of sinusoidal and rhythmic patterns during the first one hour after HI ($P<0.05$, sinusoidal pattern; vs mild and moderate groups, rhythmic pattern; vs mild group, Figure 6C). The mild HI group showed short, isolated periods of rhythmic patterns during the first two hours, but true sinusoidal patterns were not observed.

Serum AVP values were significantly increased at 1 hour after UCO in the moderate and severe group compared to controls ($P<0.005$, Figure 6A). The increase in AVP was significantly greater after severe HI than moderate HI (259 ± 100 pmol/l vs 87 ± 68 pmol/l, median ± IQR, $P<0.01$). AVP concentrations were positively correlated with the duration of sinusoidal pattern 1 hour after UCO ($r^2=0.57$, $P<0.005$, Figure 6B), but were not significantly associated with the total duration of rhythmic patterns. Finally, both the total duration of sinusoidal FHR patterns in the first 2 hours after HI ($r^2=0.47$, $P<0.005$, Figure 7), and AVP levels at 1 hour after HI ($r^2=0.61$, $P<0.0005$), were associated with loss of EEG power at day 3 after HI.

Discussion

This study evaluated the potential utility of fetal HRV as a biomarker of the severity of HI in near-term fetal sheep within the established therapeutic window for hypothermia of the first 6 hours after HI (Wassink *et al.*, 2018). VLF spectral power of fetal HRV was consistently suppressed after all severities of HI. Although the magnitude of suppression was not affected by the severity of HI, the duration of suppression was markedly prolonged by more severe HI, from 3 hours after mild HI to 12 hours after severe HI. Sample entropy, a measure of complexity in the fetal HR, showed a highly similar pattern with a rapid increase after UCO that was similar between the HI groups, with greater duration of this increase in proportion to greater severity of HI. Intriguingly, LF and HF spectral power showed a completely different profile across the groups, marked by initial suppression in the mild group, but transient normalisation for several hours in the moderate and severe groups. These differential patterns in spectral HRV power and in relative spectral power raise the possibility that more severe HI was associated with greater autonomic dysfunction.

The differential changes in HRV power in the moderate and severe groups likely in part reflected the presence of abnormal rhythmic fetal HR patterns in the early latent phase. These patterns included a mixture of sinusoidal and sinusoidal-like events. The sinusoidal pattern was seen in the early latent phase, and almost exclusively after severe HI (6/8 of fetuses exposed to severe HI, compared to 1/8 moderate and no cases of mild HI). Although the episodes of sinusoidal HR were relatively brief (200 ± 33 seconds per 10 minute period), the duration of the sinusoidal pattern in the first 2 hours after HI was associated with loss of EEG power by day 3 (r^2=0.47).

Consistent with the present study, we have previously reported that in preterm fetal sheep at 0.6 and 0.7 of gestation time domain measures of fetal HRV are suppressed for the first 2-3 hours of the latent phase after HI (George *et al.*, 2004; Yamaguchi *et al.*, 2018). Together, these previous studies and the present findings, suggest that the underlying effect of HI is suppression of background fetal HRV. However, the present study shows that this background suppression can be obscured by superimposed HR rhythms that increase measures of fetal HRV. The reader should note that there was predominance of female fetuses, particularly in the control and mild groups; further studies are needed to evaluate whether sex affects these responses (Bennet *et al.*, 2007). During the first 6 hours after UCO, EEG activity remained profoundly suppressed in all HI groups. This suppression is an actively mediated, endogenous neuroprotective response that helps to limit the spread of neural injury (Dean *et al.*, 2006; Jensen *et al.*, 2006; Yawno *et al.*, 2007; Lear *et al.*, 2014). Thus, suppression of overall fetal HRV after mild HI and of VLF power after all grades of HI is likely a function of suppressed cerebral activity, and so of autonomic activity (Yamaguchi *et al.*, 2018). We speculate that this relative shift in power of HRV towards higher frequencies in the first few hours after UCO in the moderate and severe

HI groups and the associated rhythmic HR patterns may reflect evolving brainstem injury (George *et al.*, 2004) leading to abnormal autonomic outflow.

The overall pattern of changes in the non-linear measure, SampEn, after HI were broadly similar to the changes in VLF power, albeit with an initial substantial increase rather than decrease in all three HI groups during the first three hours of the latent phase. SampEn values in the mild and moderate groups then returned to control levels, whereas in the severe group values remained elevated until 24 hours after HI, including a delayed increase between 18 and 24 hours. SampEn is a measure of complexity and regularity of the HR trace such that an increase in SampEn reflects greater complexity (Richman & Moorman, 2000). Interestingly, a combination of both decreased SampEn and suppressed time-domain measures of HRV have been observed in the early stages of neonatal sepsis (Lake *et al.*, 2002). In contrast, epileptic seizures in adults are associated with a decrease in SampEn, but an increase in time domain measures of HRV (Pavei *et al.*, 2017). In the present study, we observed a third combination during the early latent phase, of increased SampEn in association with suppression of VLF power across all three HI groups. These differential findings illustrate that SampEn has a complex relationship with both time and frequency domain measures of HRV. The patterns of SampEn and measures of HRV in the present study suggest that although total autonomic outflow was reduced during the latent phase, the remaining autonomic signalling to the SA node was comparatively more irregular or spasmodic in nature.

Pragmatically, the complexity of fetal HRV power changes over time between groups would greatly limit their use as bedside markers of HI. All HI groups were associated with a similar magnitude of VLF power suppression in the critical first 3 hours after HI. The moderate and severe HI groups showed continuing suppression of VLF for 6 and 12 hours after UCO, respectively, and reciprocally increased SampEn suggesting that more prolonged suppression of VLF power was associated with more severe neural injury. However, this would only be useful relatively late in the latent phase. Nonetheless, the analysis of relative spectral powers showed that mild HI was associated with symmetrical suppression of all three bands for the first 3 hours whereas moderate and severe HI were associated with a reduction in relative VLF power. The severe group further showed a significant but transient increase in relative LF power, which resolved to below sham control values after the first few hours. These findings indicate a shift in power away from VLF towards the higher frequency bands in both groups, that was most marked in the severe group. Alterations in relative spectral powers in the setting of suppressed VLF power may therefore be more predictive of severe injury than the absolute power alone.

The rhythmic FHR patterns observed visually after severe HI predominantly occurred at a frequency of approximately 0.04 Hz (i.e. at the boundary of the VLF and LF bands but predominantly within the LF band) and therefore likely contributed to the normalisation of LF power early after severe HI. These

patterns often met the definition for sinusoidal fetal HR patterns (American College of Obstetricians and Gynecologists, 2010). The moderate group similarly showed the appearance of rhythmic fetal HR patterns, but they were less common than after severe HI. Although these patterns had a similar frequency of oscillation, they were less likely to meet the formal definition of sinusoidal fetal HR pattern. Episodes of marked variability were also observed during the early latent phase, predominantly after severe HI, are likely at least in part contributed to increased HF power.

In addition to derangements in autonomic nervous activity, the present study raises the possibility that circulating AVP levels may contribute to these abnormal fetal HR patterns after moderate and severe HI. AVP is a significant endogenous vasopressor (Fletcher *et al.*, 2006). Consistent with this, AVP levels were significantly more elevated after severe HI than moderate HI, and levels progressively resolved from 1 hour after HI. Humoral factors are not usually thought to be major regulators of HRV, but there is evidence in fetal sheep that the combination of parasympathetic blockade (via either atropine or bilateral cervical vagotomy) and infusion of AVP can generate a rhythmic sinusoidal pattern (Murata *et al.*, 1985). The cellular mechanisms behind this are unknown, but may be related to overstimulation of myocardial AVP receptor 1A and subsequent abnormal calcium transients (Murata *et al.*, 1985). In the present study, the total duration of sinusoidal HR pattern between 0-2 hours after HI were correlated with plasma level AVP measured at one hour after HI. Unfortunately, we did not have AVP samples available before 1 hour and therefore our AVP measurements were taken a mean of 30 minutes after the peak appearance of sinusoidal activity, potentially reducing the strength of the relationship. Nonetheless, AVP levels 1 hour after HI were strongly associated with loss of EEG power by day 3 (r^2=0.61), consistent with clinical studies suggesting that AVP or its more stable co-metabolite copeptin may have value as a standalone biomarkers for HIE (Evers & Wellmann, 2016; Summanen *et al.*, 2017). Further studies are needed to understand the relationship between AVP levels after HI and neural injury.

Fetal heart rate variability in the secondary phase

The secondary phase of injury is characterized by secondary mitochondrial failure, delayed cell death and the development of post-HI seizures (Bennet *et al.*, 2010; Wassink *et al.*, 2018). Consistent with the previous finding of delayed suppression of fetal HRV in preterm fetal sheep after severe HI leading to brain stem injury (George *et al.*, 2004), in the present study, mild and moderate HI were associated with sustained recovery of fetal HRV power, whereas severe HI led to progressive loss of absolute LF and HF power from 18 hours onward. Similarly, there was a delayed increase in SampEn after severe HI at this time, suggesting that a further period of sporadic autonomic outflow occurred during the secondary phase of injury. This secondary loss of fetal HRV power was associated with a marked secondary fall in EEG power after severe HI, consistent

with the hypothesis that loss of HRV during the secondary phase reflects extensive permanent brain injury. These findings are consistent with clinical evidence that suppressed HRV between 12-48 hours of life was associated with severe HIE and worse neurodevelopmental outcome at two years of age (Goulding *et al.*, 2015). Similarly, Metzler and colleagues also reported that the degree of HRV suppression at 24-27 hours of life was associated with basal ganglia or global injury on magnetic resonance imaging and death (Metzler *et al.*, 2017).

In the present study, both mild and moderate HI were associated with slow recovery of EEG power to control values, although to a lesser degree than after severe HI. This suggests that long-term changes in HRV are not sensitive biomarkers of milder HI injury. This difference mostly likely reflects initial preservation of blood flow to the brainstem compared to the cortex during HI (Jensen *et al.*, 1991).

Perspectives and significance

Clinical studies in infants with moderate to severe HIE have emphasized that suppression of HRV in the secondary phase is associated with poor outcomes. This association appears to be mediated by extensive, permanent brain injury. By contrast, in the early latent phase (the first 3 hours) after UCO, it is striking that the most robust suppression of HRV power was observed after mild HI. Both more prolonged suppression of fetal HRV power and reciprocally increased SampEn were associated with the severity of HI in the present study. This combination suggests that although autonomic function is suppressed, residual autonomic function is more irregular or spasmodic likely reflecting prolonged dysfunction of brain stem cardiovascular centres. Pragmatically, the time course of these changes must greatly limit their utility as biomarkers of HI in the early latent phase.

By contrast, the presence of aberrant HR patterns, particularly sinusoidal patterns, in the first 2 hours was seen frequently after moderate to severe HI and was significantly associated with fetal AVP levels, changes in the distribution of HRV power, and subsequent loss of EEG power at day 3 after HI. Further, AVP levels at 1 hour after HI were also strongly associated with loss of EEG power. These findings suggest that changes in the pattern of HR and AVP levels may have predictive utility in the first few hours after exposure to HI.

Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

These experiments were conducted in the Fetal Physiology and Neuroscience Group laboratory, at the University of Auckland. AJG, CAL, EM, TI and LB conceived the hypotheses, experimental design and analysis protocols for the study. MK, CAL, PPD, JOD, YM and AJG were responsible for data collection. MK, CAL and MJB performed the physiological analysis. MK drafted the manuscript. MK and CAL contributed equally to this study and qualify as equal first authors. All authors were involved in data interpretation and critically reviewed the manuscript. All authors listed qualify for authorship and approved the final version of the manuscript submitted for publication.

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Table

Table 1. Fetal arterial pH, blood gases, and metabolites before, during and after umbilical cord occlusion

Group	Baseline	Occlusion	+1h	+2h	+4h	+6h	+24h	+48h	+72h
pH									
control	7.39 ± 0.01	7.39 ± 0.02	7.39 ± 0.03	7.38 ± 0.04	7.38 ± 0.05	7.39 ± 0.06	7.38 ± 0.07	7.37 ± 0.08	7.36 ± 0.09
mild	7.36 ± 0.02	6.97 ± 0.02$^{\phi}$	7.32 ± 0.03	7.38 ± 0.03	7.37 ± 0.01	7.37 ± 0.01	7.38 ± 0.01	7.37 ± 0.02	7.37 ± 0.02
moderate	7.37 ± 0.01	6.89 ± 0.02‡	7.25 ± 0.03$^{\#}$	7.29 ± 0.04	7.35 ± 0.02	7.37 ± 0.01	7.40 ± 0.01	7.40 ± 0.02	7.39 ± 0.01
severe	7.39 ± 0.01	6.92 ± 0.01†	7.21 ± 0.02§,†	7.25 ± 0.02§	7.28 ± 0.05	7.31 ± 0.05	7.39 ± 0.03	7.41 ± 0.01	7.40 ± 0.01
P_{aCO2} (mmHg)									
control	53.2 ± 1.6	52.8 ± 1.7	53.1 ± 1.6	48.6 ± 2.6	47.4 ± 1.8	52.9 ± 1.8	52.0 ± 1.8	53.0 ± 3.0	53.3 ± 1.5
mild	46.9 ± 2.5	112.7 ± 9.7$^{\phi}$	47.4 ± 2.9	44.8 ± 1.7	46.1 ± 1.9	44.6 ± 4.0	45.5 ± 2.4	48.1 ± 2.5	49.3 ± 2.9
moderate	54.5 ± 2.0	143.4 ± 4.4$^{\#,\ddagger}$	54.5 ± 1.9	48.0 ± 1.6	48.3 ± 1.2	51.8 ± 1.0	53.4 ± 1.7‡	50.6 ± 1.5	52.2 ± 1.8
severe	51.8 ± 1.4	133.1 ± 4.4†	54.8 ± 1.4	48.0 ± 1.3	47.6 ± 1.6	52.1 ± 2.3	48.1 ± 1.3	48.6 ± 2.2	50.7 ± 1.7
P_{aO2} (mmHg)									
control	23.1 ± 0.9	23.6 ± 1.0	22.9 ± 0.8	23.7 ± 1.3	22.7 ± 1.2	22.1 ± 1.4	23.8 ± 1.1	22.2 ± 0.8	21.6 ± 0.7
mild	23.1 ± 1.6	6.6 ± 0.7$^{\phi}$	23.8 ± 2.7	21.8 ± 2.0	19.5 ± 1.6	21.9 ± 2.8	19.1 ± 2.2	19.8 ± 1.9	22.3 ± 0.8
moderate	23.5 ± 1.3	6.2 ± 1.4$^{\#}$	25.8 ± 1.7	25.1 ± 1.3	24.5 ± 0.9	22.9 ± 1.1	23.4 ± 1.0	25.8 ± 1.9	26.1 ± 1.7
severe	21.2 ± 0.8	4.6 ± 0.5†	24.5 ± 1.3	22.1 ± 1.9	19.6 ± 1.2¶	18.1 ± 1.9	19.1 ± 1.2	25.4 ± 1.1	26.3 ± 1.4
BE									
control	5.5 ± 1.1	5.5 ± 1.0	5.3 ± 0.8	2.3 ± 1.4	2.1 ± 1.3	5.5 ± 1.2	3.9 ± 1.2	3.8 ± 1.6	3.6 ± 2.0
mild	1.1 ± 2.1	-8.2 ± 1.3$^{\phi}$	-3.1 ± 1.7$^{\phi}$	0.3 ± 2.1	-0.6 ± 0.7	-1.7 ± 1.8	1.3 ± 1.1	2.3 ± 2.6	2.1 ± 2.1
moderate	4.5 ± 0.8	-9.0 ± 1.0$^{\#}$	-5.5 ± 1.0$^{\#}$	-2.3 ± 0.5	0.6 ± 2.1	3.1 ± 1.4	6.3 ± 0.5	4.8 ± 0.5	4.8 ± 0.4
severe	4.4 ± 0.5	-8.7 ± 0.5†	-6.5 ± 0.7†	-5.9 ± 1.4†,§	-4.2 ± 2.6	0.9 ± 1.9	3.4 ± 2.0	5.1 ± 0.9	5.3 ± 0.6
Hct (%)									
control	30.6 ± 0.7	30.8 ± 0.9	30.2 ± 1.0	28.7 ± 0.7	27.8 ± 0.7	29.8 ± 1.0	28.4 ± 1.0	29.0 ± 2.1	30.8 ± 1.1
mild	31.1 ± 1.2	36.8 ± 1.3	32.6 ± 1.8	32.6 ± 1.3	32.4 ± 3.4	32.2 ± 4.2	30.3 ± 2.3	29.0 ± 1.8	28.9 ± 1.2
moderate	30.1 ± 2.0	33.4 ± 1.5	30.1 ± 1.6	27.9 ± 1.3	27.9 ± 1.7	29.8 ± 1.7	29.1 ± 1.5	29.0 ± 1.7	28.7 ± 2.2
severe	31.3 ± 1.6	34.5 ± 1.7	33.9 ± 1.5	31.6 ± 1.6	30.4 ± 1.5	32.7 ± 2.4	34.0 ± 3.0	33.4 ± 2.3	30.0 ± 1.7
O_{2ct} (mmol L^{-1})									
control	4.2 ± 0.1	4.2 ± 0.1	4.0 ± 0.1	3.9 ± 0.1	3.6 ± 0.1	4.0 ± 0.3	4.0 ± 0.2	3.7 ± 0.1	3.8 ± 0.3
mild	3.9 ± 0.4	0.4 ± 0.1$^{\phi}$	3.8 ± 0.4	3.7 ± 0.6	3.3 ± 0.3	3.8 ± 0.0	3.3 ± 0.5	3.4 ± 0.3	3.6 ± 0.1
moderate	4.0 ± 0.3	0.5 ± 0.1$^{\#}$	4.0 ± 0.4	3.7 ± 0.3	3.9 ± 0.3	3.9 ± 0.3	4.3 ± 0.2	4.2 ± 0.3	4.1 ± 0.3
severe	4.2 ± 0.2	0.5 ± 0.0†	4.2 ± 0.3	3.3 ± 0.3	3.0 ± 0.3	3.1 ± 0.4	3.8 ± 0.6	5.0 ± 0.4†,§	4.6 ± 0.4
Lactate (mmol L^{-1})									
control	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
mild	0.7 ± 0.1$^{\phi}$	4.5 ± 0.7$^{\phi}$	3.3 ± 0.6	2.4 ± 0.9	1.8 ± 0.7	1.8 ± 0.8	1.1 ± 0.2	1.7 ± 0.6	0.9 ± 0.2
moderate	1.2 ± 0.1‡	6.5 ± 0.7$^{\#}$	6.9 ± 0.7$^{\#,\ddagger}$	5.9 ± 1.0$^{\#}$	5.2 ± 1.2	4.7 ± 0.9	1.6 ± 0.2	1.3 ± 0.1	1.1 ± 0.2
severe	1.1 ± 0.1§	6.1 ± 0.2†	6.6 ± 0.3†,§	6.3 ± 0.7†	6.4 ± 1.5†	5.8 ± 1.5†	5.2 ± 1.2†,§,¶	1.4 ± 0.1	1.1 ± 0.1
Glucose (mmol									

L^{-1})									
control	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
mild	0.6 ± 0.1$^{\phi}$	0.6 ± 0.2	0.9 ± 0.1	0.9 ± 0.0	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	0.6 ± 0.1
moderate	0.9 ± 0.1	1.1 ± 0.2	1.5 ± 0.1	1.3 ± 0.1$^{\#}$	1.3 ± 0.1$^{\#,\ddagger}$	1.6 ± 0.1$^{\#,\ddagger}$	1.3 ± 0.1	1.3 ± 0.1‡	1.0 ± 0.1‡
severe	0.8 ± 0.1	1.1 ± 0.1	1.7 ± 0.2†,§	1.2 ± 0.1	1.1 ± 0.1	1.4 ± 0.1	1.8 ± 0.2†,§	1.5 ± 0.2†,§	1.0 ± 0.1

Samples during umbilical cord occlusion were taken at either 8 min (10 min UCO) or 12 min (for the 15 min and 15+ min UCO protocols) after the start of UCO. Control: n=5; mild: n=6; moderate: n=8; severe: n=8, P_{aCO2}: partial pressure of carbon dioxide, P_{aO2}: partial pressure of oxygen, BE: base excess, Hct: haematocrit, O_{2ct}: oxygen content. $^{\phi}P < 0.05$, sham vs. mild; $^{\#}P < 0.05$, sham vs. moderate; $^{\dagger}P < 0.05$, sham vs. severe; $^{\ddagger}P < 0.05$, mild vs. moderate; $^{\S}P < 0.05$, mild vs. severe; $^{\P}P < 0.05$, moderate vs. severe.

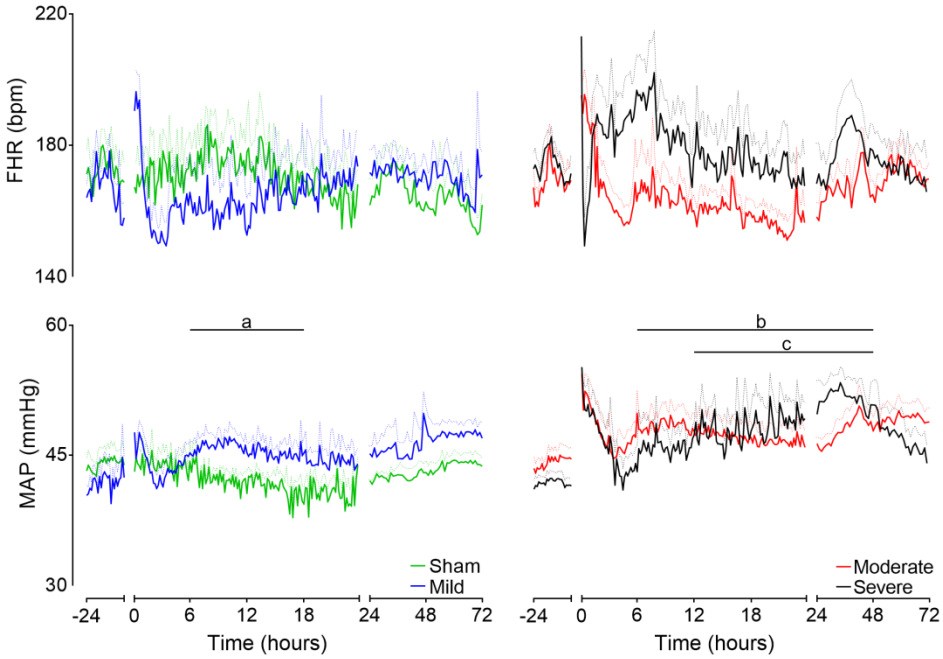

Figure 1. Fetal heart rate and mean arterial pressure from 24 hours before until 72 hours after hypoxia-ischaemia.

Time sequence of changes in fetal heart rate (HR) and mean arterial pressure (MAP) from 24 hours before until 72 hours after hypoxia-ischaemia induced by umbilical cord occlusion (UCO). Time 0 represents the end of UCO. Data during UCO are not shown. Data are 10 min mean ± SEM during the first 24 hours after occlusion and 1-h mean ± SEM at all other time points. Left panels show the sham control (green, n=5) and mild (blue, n=6) HI groups. The right hand panels show the moderate (red, n=8) and severe (black, n=8) HI groups. Fetal HR: fetal heart rate, MAP: mean arterial pressure. a $P<0.05$, sham vs. mild; b $P<0.05$, sham vs. moderate; c $P<0.05$, sham vs. severe

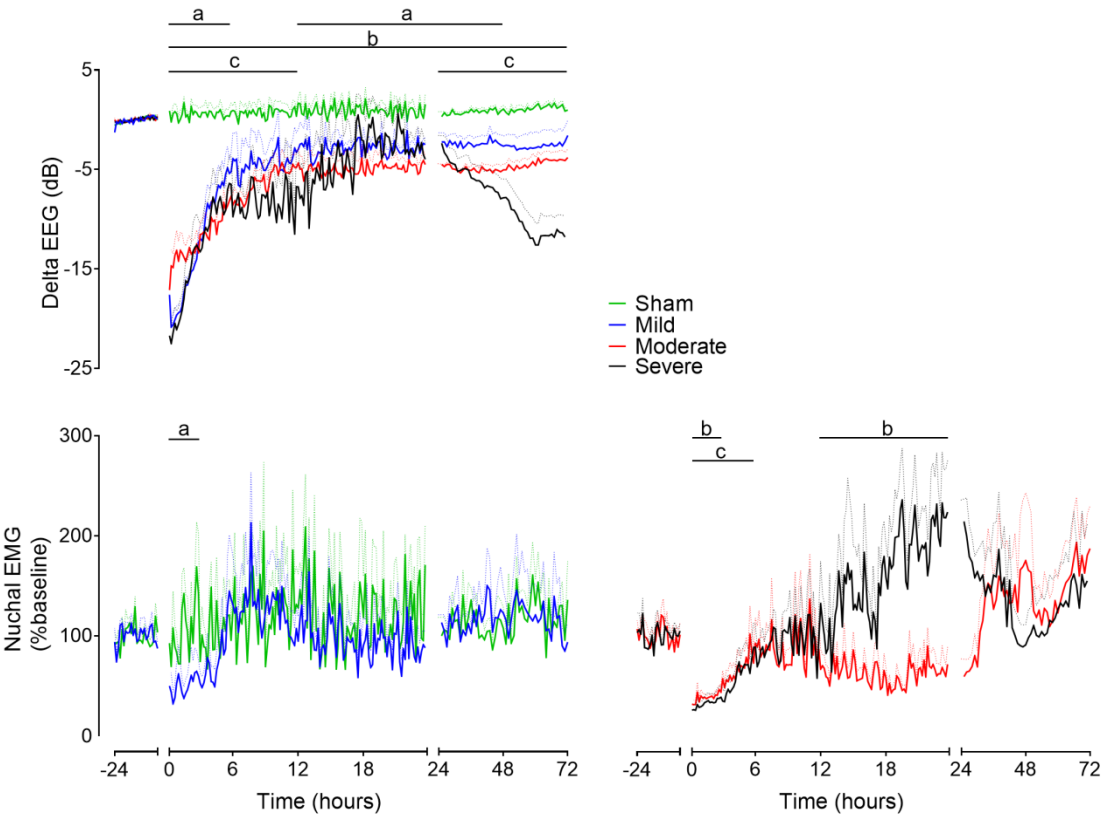

Figure 2. EEG power and nuchal electromyographic activity after hypoxia-ischaemia.

Time sequence of changes in EEG power and nuchal electromyographic (EMG) activity from 24 hours before until 72 hours after sham occlusion (green, n=5), or mild (blue, n=6) or moderate (red, n=8) or severe (black, n=8) hypoxia-ischaemia induced by umbilical cord occlusion. a $P<0.05$, sham vs. mild; b $P<0.05$, sham vs. moderate ; c $P<0.05$ sham vs. severe.

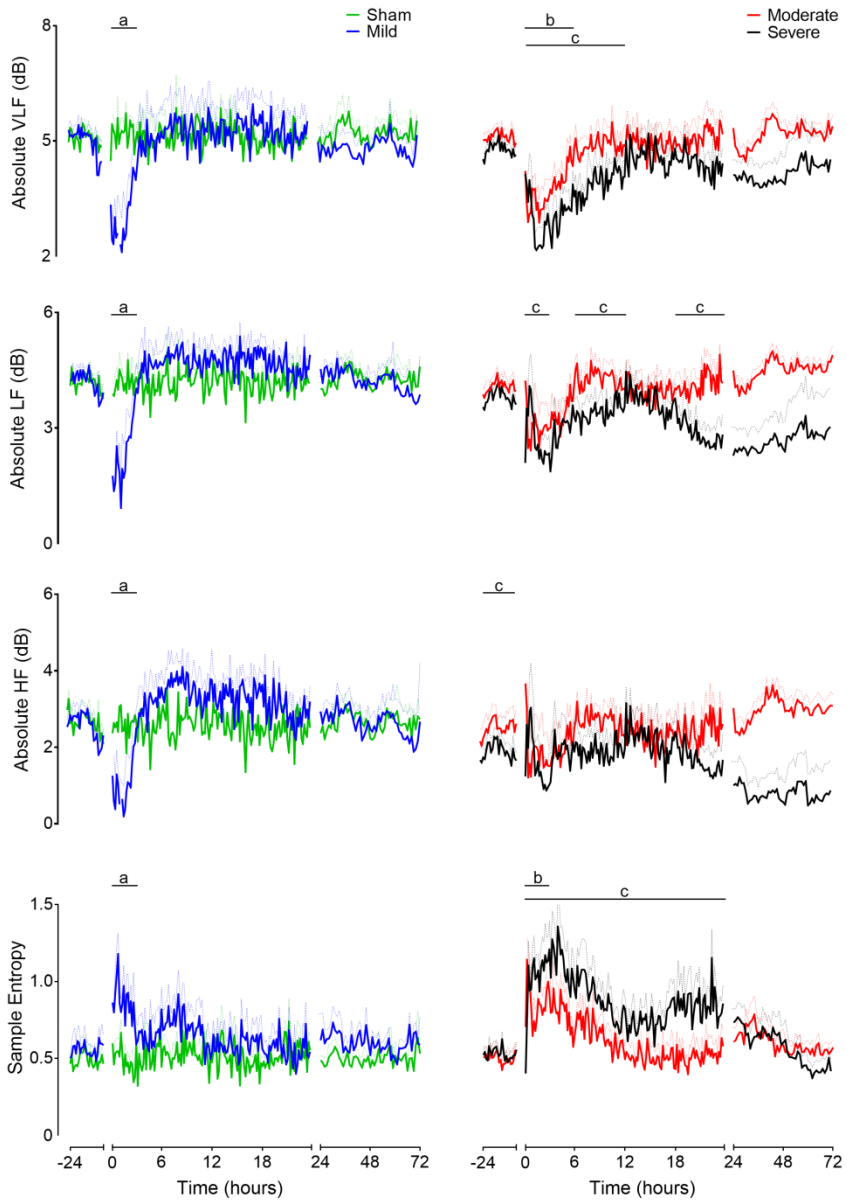

Figure 3. Spectral power and sample entropy of fetal heart rate variability after hypoxia-ischaemia.

Time sequence of changes in fetal spectral power and sample entropy of heart rate variability from 24 hours before until 72 hours after sham occlusion (green, n=5), or mild (blue, n=6) or moderate (red, n=8) or severe (black, n=8) hypoxia-ischaemia induced by umbilical cord occlusion. The Left panels present changes in frequency bands of the HI sham and mild groups, showing initial suppression of all frequency bands after mild HI. The right panels present the moderate and severe HI groups, showing secondary suppression in LF and HF in the secondary phase after severe but not moderate HI. All HI groups showed increased SampEn after HI, which was significantly more prolonged after severe HI. a $P<0.05$, sham vs. mild; b $P<0.05$, sham vs. moderate; c $P<0.05$, sham vs. severe

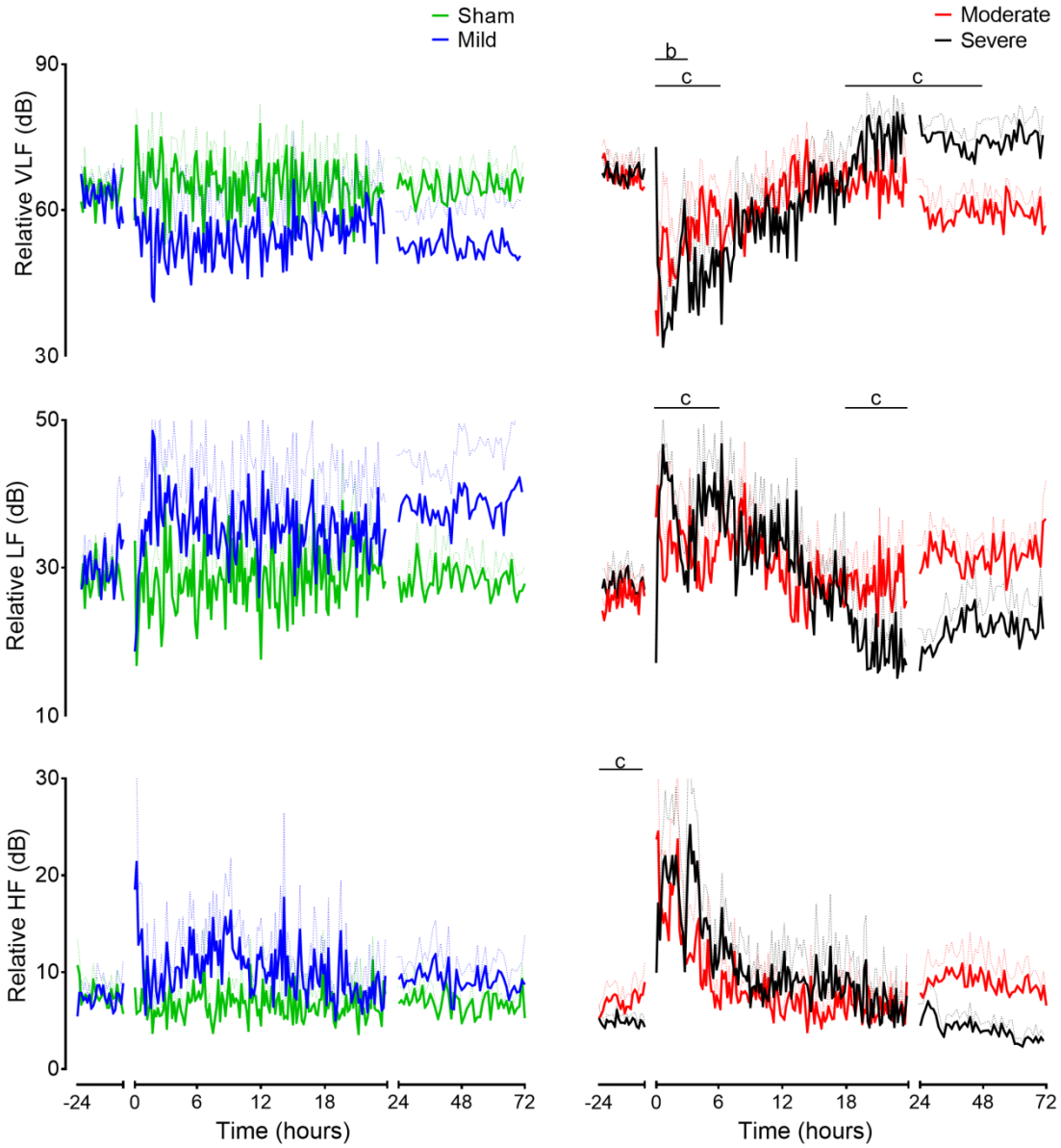

Figure 4. Relative spectral power fetal heart rate variability after hypoxia-ischaemia.

Time sequence of changes in the proportions of very low frequency (VLF), low frequency (LF) and high frequency (HF) fetal spectral power (decibels, db) after severe hypoxia-ischaemia (HI) induced by umbilical cord occlusion. Sham control (green, n=5) and Mild (blue, n=6) HI shown on the left. Moderate (red, n=8) and severe (black, n=8) HI are shown on the right. Mild HI was not associated with any significant change in the relative proportions of the spectral power bands whereas both moderate and severe groups showed initial suppression in VLF only. Severe HI was associated with delayed onset of suppression of LF and HF. b $P<0.05$, sham vs. moderate; c $P<0.05$, sham vs. severe.

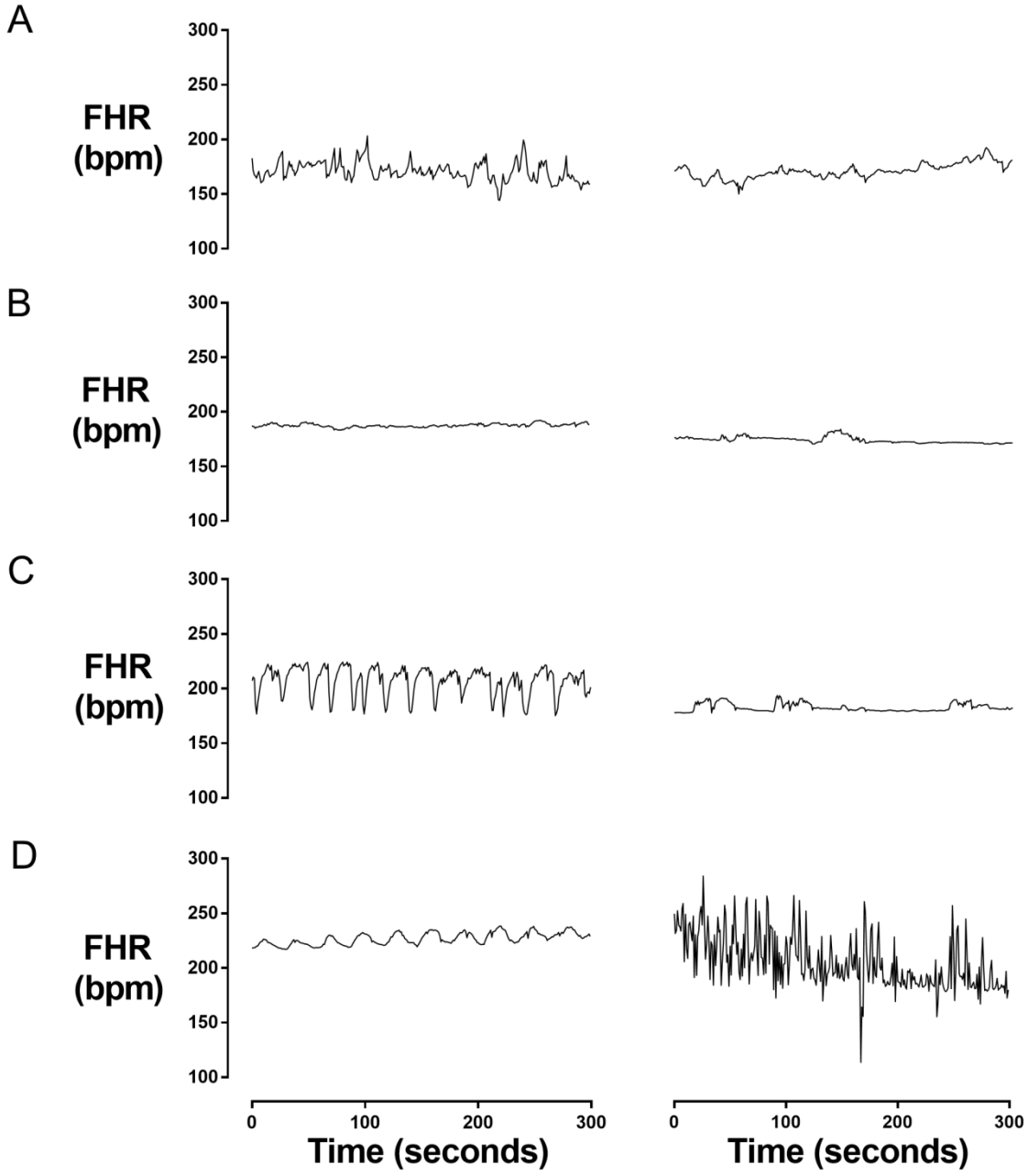

Figure 5. Examples of fetal heart rate patterns in the first 2 hours after hypoxia-ischaemia.

Examples of 5 minute intervals of fetal heart rate (FHR) patterns in the first 2 hours after A. sham controls, and B. mild, C. moderate, D. severe hypoxia-ischaemia induced by umbilical cord occlusion. Note marked suppression of FHR variation after mild HI (Panel B) compared to sham controls (Panel A). Moderate HI was typically associated with a mixture of rhythmic patterns (Panel C, left) and suppression (Panel C, right). Severe HI was most often associated with periods of sinusoidal FHR activity (Panel D, left), and increased variability (Panel D, right).

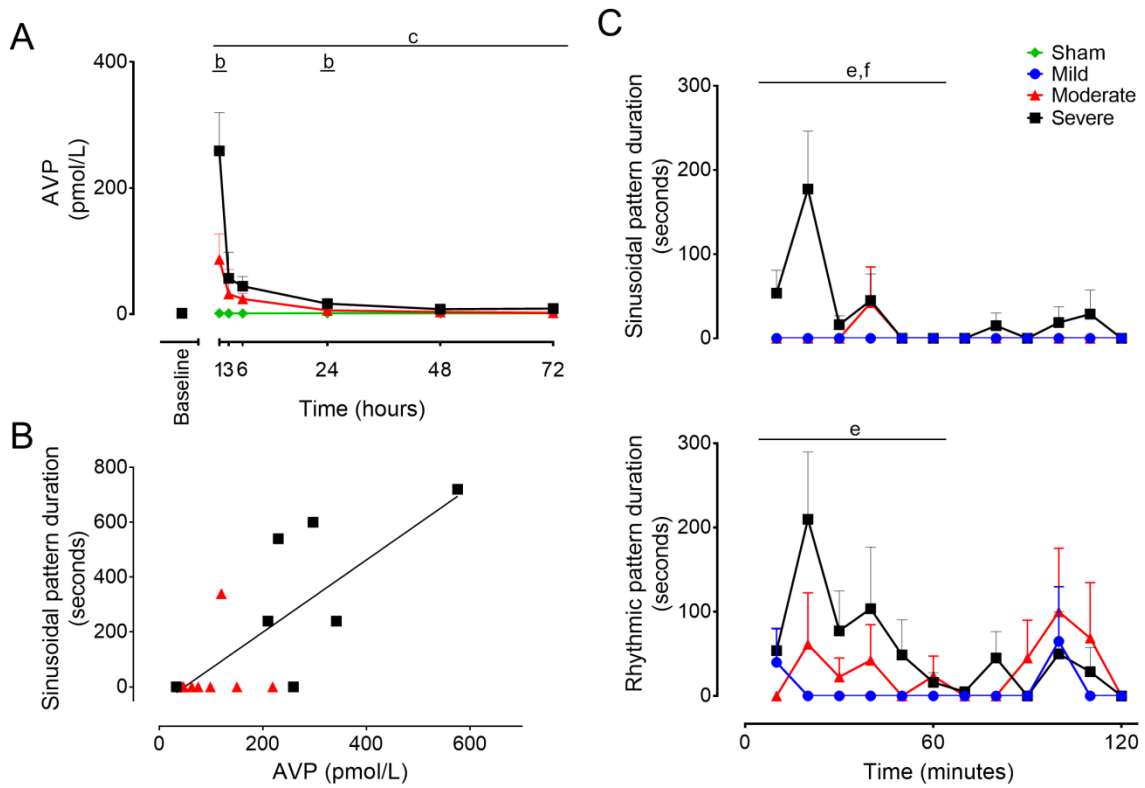

Figure 6. Fetal arginine vasopressin levels and fetal heart rate patterns after hypoxia-ischaemia.

Panel A. Time sequence of changes in fetal serum arginine vasopressin (AVP) levels after sham control (green, n=5), moderate (red, n=8) or severe (black, n=7) hypoxia-ischaemia induced by umbilical cord occlusion (UCO). Time 0 = the end of UCO. Panel B. Correlation between total cumulative duration of fetal sinusoidal HR patterns in the first 2 hours and serum AVP concentrations at 1 hour after UCO (r^2=0.57, P<0.005). Moderate (red triangle, n=8), severe (black square, n=7). Panel C. Time sequence of changes in time per 10-minute period of the sinusoidal pattern (top) and total rhythmic patterns from 0 to 2 hours after umbilical cord duration (bottom). Mild (blue, n=6), moderate (red, n=8) and severe (black, n=8). b P<0.05, sham vs. moderate; c P<0.05, sham vs. severe; e P<0.05, mild vs. severe; f P<0.05, moderate vs. severe.

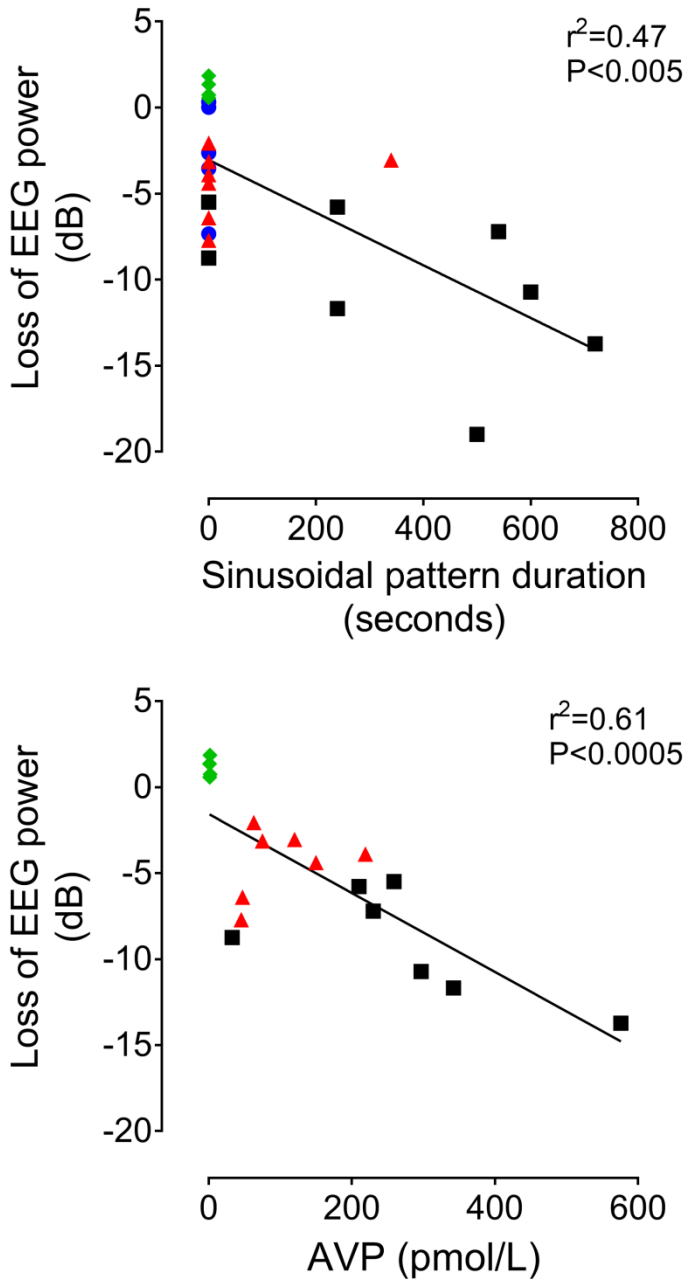

Figure 7. Correlation between duration of sinusoidal pattern and plasma arginine vasopressin levels and loss of brain activity at day 3.

Electroencephalographic (EEG, dB) power at day 3 is shown as changes from baseline. Top panel; Sinusoidal pattern duration within 2 hours after umbilical cord occlusion (UCO) was associated with loss of EEG power at day 3 after HI. Sham (green diamonds, n=4), mild (blue circles, n=5), moderate (red triangles, n=7), severe (black squares, n=8). Bottom panel; serum arginine vasopressin (AVP) AVP levels at 1 hour after moderate to severe HI was correlated with EEG power at day 3. Sham (green diamonds, n=4), moderate (red triangles, n=7), severe (black squares, n=7).