Fetal Heart Rate Variability and Brainstem Injury after Asphyxia in Preterm Fetal Sheep

Sherly George,* Alistair J. Gunn,⁎‡ Jenny A. Westgate,† Christine Brabyn,⁎ Jian Guan,# Laura Bennet,*†

⁎The Department of Physiology, †Depts of Obstetrics and Gynaecology and ‡Paediatrics and #The Liggins Institute, Faculty of Medicine and Health Science, The University of Auckland, Private Bag 92019, Auckland, New Zealand.

Running Title. FHR variability after preterm asphyxia

Corresponding Author.

Dr Laura Bennet,
Department of Physiology,
The University of Auckland,
Private Bag 92019, Auckland,
New Zealand.
E-mail: l.bennet@auckland.ac.nz
Phone: (64-9) 373 7599, ext. 84890
Fax: (64-9) 373 7499
Abstract

This study was undertaken to determine the mechanisms mediating changes in fetal heart rate variability (FHRV) during and after exposure to asphyxia in the premature fetus. Preterm fetal sheep at 0.6 gestation (91±1 days, term is 147 days) were exposed to either sham occlusion (n=10) or to complete umbilical cord occlusion for either 20 (n=7) or 30 minutes (n=10). Cord occlusion led to a transient increase in FHRV with abrupt body movements which resolved after 5 min. In the 30 min group there was a marked increase in FHRV in the final 10 min of occlusion, related to abnormal atrial activity. After reperfusion FHRV in both study groups was initially suppressed and progressively increased to baseline levels over the first four hours of recovery. In the 20 min group this improvement was associated with return of normal EEG activity and movements. In contrast, in the 30 min group the EEG was abnormal with epileptiform activity superimposed on a suppressed background which was associated with abnormal fetal movements. As the epileptiform activity resolved FHRV fell and became suppressed for the remainder of the study. Histological assessment after 72 h demonstrated severe brainstem injury in the 30 min group, but not in the 20 min group. In conclusion, during early recovery from asphyxia epileptiform activity and associated abnormal fetal movements related to evolving neural injury can cause a confounding transient increase in FHRV, which mimics the normal pattern of recovery. However, chronic suppression of FHRV was a strong predictor of severe brainstem injury.

Keywords. Asphyxia, fetal heart rate variability, brainstem injury
INTRODUCTION

Exposure of prematurely delivered infants to perinatal asphyxia is associated with a marked increase in morbidity and mortality (25, 27). Even moderate acidosis is associated with later impairment of cognitive performance (18, 51). A reduction in fetal heart rate variability (FHRV), particularly when it is combined with other fetal heart rate abnormalities, is reported to be an important indicator of fetal hypoxia and developing acidemia both in the term (58) and preterm fetus (29). Perhaps surprisingly, however, many clinical studies have suggested there is either a weak or no relationship between FHRV and Apgar scores or cord acid-base measures during labor (37, 43). Indeed, the initial response to acute experimental hypoxemia or repeated asphyxia in the term fetus is an increase in FHRV rather than a decrease (9, 24, 33, 55); typically FHRV then becomes suppressed if the insult is continued or repeated (16, 20, 33, 55).

These experimental data relate to the mature fetus. The sympathetic and parasympathetic control of fetal heart rate activity and variability progressively mature in the last third of pregnancy (7), with a parallel maturation of baroreceptor responses, which also influence FHRV (30). In contrast, preterm fetuses have lower basal FHRV than at term (6), and do not show the cyclical changes in FHRV related to behavioral state that are seen near-term (11, 52). It is not known how this immaturity affects FHRV responses to asphyxia. A further important clinical concern is that since brainstem nuclei, including the dorsal motor nucleus of the vagus and the nucleus ambiguus, have a central role in cardiac control (12, 13), antenatal asphyxial neural injury may itself lead to a confounding loss of FHRV (39, 47). This concept is supported by reports of reduced FHRV in preterm infants with evidence of cerebral injury or suppression (38, 41).

Therefore, the purpose of the current study was to dissect the effects of asphyxia and of neural
injury on changes in FHRV during and following exposure to asphyxia, using an established model of complete umbilical cord occlusion in the near mid-gestation fetal sheep (4). The duration of cord occlusion was varied to produce asphyxia with or without severe neural injury.
METHODS

Animal preparation

Twenty seven Romney/Suffolk fetal sheep were instrumented at 86-89 days of gestation (term = 147 days) as previously described (4). All procedures were approved by the Animal Ethics Committee of the University of Auckland. Food, but not water was withdrawn 18 h before surgery. Ewes were given 5 mls of Streptopen (Procaine Penicillin (250,000 IU) and Dihydrostreptomycin (250mg/ml), Pitman-Moore, Wellington, New Zealand) intramuscularly for prophylaxis 30 min prior to the start of surgery. Anesthesia was induced by intravenous (I.V.) injection of Saffan (Alphaxalone and Alphadolone; 3mg/kg, Schering-Plough Animal Health Ltd, Wellington, New Zealand), and general anesthesia maintained using 2-3% halothane in O$_2$. The depth of anesthesia and maternal respiration were constantly monitored by trained anesthetic staff. Under anesthesia a 20 gauge I.V. catheter was placed in a maternal front leg vein, and the ewes were placed on a constant infusion saline drip to maintain maternal fluid balance.

Using sterile techniques, catheters were placed in the left fetal femoral artery and vein, right axillary artery and the amniotic sac. Two pairs of EEG electrodes (AS633-5SSF, Cooner Wire Co., Chatsworth, CA, USA) were placed on the dura over the parasagittal parietal cortex (5 mm and 10 mm anterior to bregma and 5 mm lateral) and secured with cyanoacrylate glue. A reference electrode was sewn over the occiput. Electrodes were placed in the nuchal muscle to measure electromyogram (EMG) activity, and electrocardiogram (ECG) electrodes were sewn across the chest to record fetal heart rate (FHR). An inflatable silicone occluder was placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, CA, USA). All fetal leads were exteriorized through the maternal flank and a maternal long saphenous vein was catheterized to provide access for post-operative care and euthanasia. Antibiotics (80mg
Gentamicin, Rousell, Auckland, New Zealand) were administered into the amniotic sac prior to closure of the uterus.

A period of 4-6 days post-operative recovery was allowed before experiments commenced, during which time antibiotics were administered daily for 5 days I.V. to the ewe (600mg 600mg Benzylpencillin Sodium (Crystapen) and 80mg Gentamicin). Fetal catheters were maintained patent by continuous infusion of heparinized isotonic saline (10U/ml at 0.15ml/h), and the maternal catheter maintained by daily flushing.

**Experimental procedures**

*Recordings*

Fetal arterial blood pressure, corrected for maternal movement by subtraction of amniotic fluid pressure (Novatrans II, MX860; Medex Inc., Hilliard, OH, USA)(26), ECG, EEG and EMG were recorded continuously. The blood pressure signal was collected at 64 Hz and low pass filtered at 30 Hz. The raw ECG was analog filtered between 0.05 and 80 Hz and digitized at 512 Hz (55). The nuchal EMG signal was band-pass filtered between 100 Hz and 1kHz, the signal was then integrated using a time constant of 1 sec. The EEG signal was low-pass filtered at 30 Hz, and then the intensity spectrum was extracted (57). For data presentation, the total EEG intensity (power) was normalized by log transformation (dB, 20 x log (intensity)), and data from left and right EEG electrodes were averaged to give mean total EEG activity. Data were collected by computer and stored to disk for off-line analysis.

*Experimental protocol*

Experiments were conducted at 89-92 days gestation. Fetuses were randomly assigned to either the sham occlusion group (n=10), 20 min occlusion group (n=7), or 30 min occlusion group (n=10). Fetal asphyxia was induced in the occlusion groups by rapid inflation of the
umbilical occluder with sterile saline of a defined volume known to completely inflate the occluder (4). Successful occlusion was confirmed by observation of an immediate sharp rise in fetal mean arterial blood pressure (MAP) and a rapid fall in FHR in all cases. All fetuses in the occlusion groups survived occlusion. Fetal arterial blood samples were taken at 15 min prior to occlusion (or sham occlusion), 5 and 15 min of occlusion for the 20 min occlusion group, and 25 min during the period of occlusion (for the sham occlusion and 30 min group), followed by samples at 1, 6, 24, 48 and 72 h after release of occlusion for pH and blood gas determination (Ciba-Corning Diagnostics 845 blood gas analyzer and co-oximeter, MA., USA) and for glucose and lactate measurements (YSI model 2300, Yellow Springs, Ohio, USA). Fetuses were studied for three days post-asphyxia and on completion of the experiment the ewes and fetuses were killed by an overdose of sodium pentobarbitone (9 g I.V. to the ewe: Pentobarb 300, Chemstock International, Christchurch, New Zealand).

Histology Tissue Preparation

Fetal brains were perfusion fixed in situ with normal saline followed by 500 ml of 10% phosphate buffered formalin. Following removal from the skull, tissue was fixed for a further 5-6 days before processing and embedding using a standard paraffin tissue preparation (17). Neuronal loss was evaluated by light microscopy on 8-µm thick coronal sections stained with thionin and acid fuchsin by an assessor masked to the treatment group (17). The proportion of neurons showing ischemic cell change in multiple pre-assigned areas was scored on a 6 point scale: 0 = no dead neurons; 5= >0–10%; 30= >10–50%; 70= >50–90%; 95= 90–<100%; and 100 = 100% dead neurons. Average scores were calculated for each region (17).

TUNEL staining

Terminal deoxynucleotidyl transferase (TdT) – mediated dUTP- biotin nick-end labeling
(TUNEL) was performed on parallel sections to further validate the extent of cell death (3). Briefly the coronal sections (8µm) adjacent to the sections used for neuronal loss scores were pretreated for 15 min with Proteinase K (40 µg/ml; Sigma Chemical, St. Louis, MO, USA), washed in phosphate buffered saline (PBS), then kept for 10 min with methanol containing 1% H₂O₂ to block non-specific peroxidase activity. Sections were then washed again in PBS and incubated for 5 min with TdT buffer (GIBCO-BRL, Life Technologies, Gaithersburg, MD, USA). DNA fragments were labeled with TdT and biotin-14-dATP (Gibco-BRL) for 1 h at 37°C. Subsequently, sections were washed in SSC buffer and incubated for 2 h with ABC reagent (Vector Laboratories, Burlingame, CA, USA). After washing, the sections were developed with diaminobenzidine tetrahydrochloride (DAB) substrate. Sections were dehydrated in graded alcohols and cover-slipped with mounting medium. A section that was pretreated with DNase 1 (Sigma) to nick all DNA served as a positive control. A negative control slide was obtained with the omission of TdT from the incubation solution. The sections were then counterstained with thionin. The data were assessed and photographed using light microscopy (Nikon E800, Tokyo, Japan).

**Data analysis**

Off-line analysis of the physiological data was performed using customized Labview programmes (National Instruments, Austin, Texas, USA). Heart rate variability between occlusions was calculated as described by Dawes and colleagues (10), to obtain the mean minute range (MMR, the difference between the maximum and minimum RR intervals every minute). FHR variability was not measured during accelerations or decelerations of \( \geq 10 \) bpm for one min or \( \geq 20 \) bpm for \( >30 \) sec (10); in particular the initial FHR deceleration at the onset of occlusion and the FHR rebound during release of cord occlusion were excluded from analysis.
The raw EEG was assessed for epileptiform activity; specifically the presence of spikes and sharp waves (i.e. epileptiform transients). A spike was defined as having a sharp outline and duration of less than 70 msec. Sharp waves were assessed as transient high frequency events of moderate to high amplitude (50 to 200µV), single or repeated mono- or diphasic transients lasting 100 to 250 msec, typically superimposed on a flat EEG background (44, 45). In the 30 min occlusion group we further evaluated the contribution of body movements to the post-asphyxial FHRV responses of the fetuses by comparing fetuses in whom EMG activity returned by 24 h after the end of occlusion with those in whom it did not.

Data were analyzed using SPSS for windows (SPSS, Chicago, Il, USA). For the analysis of the occlusion and one hour post-occlusion period, data were compared to the mean of the hour prior to occlusion (baseline). For the analysis of the long-term recovery data (1-72 h post-occlusion) the baseline period was taken as the mean of the 12 h before occlusion. For between group comparisons two way analysis of variance for repeated measures was performed. When statistical significance was found between groups or between group and time analysis of covariance (ANCOVA) was used to compare selected time points, using the baseline control periods prior to occlusion as a covariate. Statistical significance was accepted when $P<0.05$. 
RESULTS
Cardiovascular, cerebrovascular, electrophysiology and blood gas data have been previously reported for a subset of the 30 min and control group fetuses (4).

Blood composition measurements
Umbilical cord occlusion, but not sham occlusion, was associated with the development of mixed respiratory and metabolic acidosis which was more severe in the 30 min group than in the 20 min group (fetal arterial pH, blood gases and lactate are shown in Table 1). There was a significant fall in glucose in both groups during occlusion (0.4±0.1 and 0.5±0.1 mmol/l in the 20 and 30 min groups respectively, vs. 1.1±0.1 mmol/l in the control group, P<0.001 both groups). During recovery there were no significant differences between the 20 min and control groups. There was a significant increase in glucose in the 30 min group at 72 h only (1.3±0.1 vs. 1.0±0.1 mmol/l, P<0.05).

Cardiovascular and FHRV responses during occlusion
There was no significant difference between groups in the baseline period. Asphyxia was associated with profound hypotension and bradycardia in both occlusion groups. FHR rapidly fell at the onset of occlusion in both groups to a nadir of 66.0±3.4 beats per minute (bpm) in the 20 min group and 53.8±3.0 bpm in the 30 min group (vs. 193.0±1.9 bpm in the control group, P<0.001, Fig. 1). During the immediate post-occlusion phase FHR was significantly elevated between 7 and 12 min (P<0.001 both groups, Fig.1). MAP was initially significantly elevated in both groups during the first five min (P<0.001), but then progressively fell and at the end of occlusion was 13.8±0.9 mmHg in the 20 min group and 11.0±0.8 mmHg in the 30 min group vs. 34.3±1.0 mmHg in the controls (P<0.001 compared with both occlusion groups, Fig.1). MAP rapidly recovered after release of occlusion, with a transient period of
hypertension observed between 6-10 min post-occlusion in both groups ($P<0.001$, Fig.1).

FHRV measured as the MMR showed an initial significant increase in both the 30 min and the 20 min groups between 3 and 5 min after the onset of occlusion ($P<0.001$, Fig.1). This then resolved until around 15 min in both groups, with significant suppression between 11 and 13 min ($P<0.05$ both groups), before increasing again. In the 30 min group FHRV was significantly different to control in the last 5 min of occlusion ($P<0.001$ vs. control, Fig. 1).

Analysis of the ECG signal of the 30 min group showed that during the last 5 min, in 5 of the 7 fetuses, that there was an altered ECG pattern consisting of bigeminy, periods of fast runs of beats, interspersed with periods of asystole in any one minute (Fig. 2A).

**EEG activity and fetal behavior during occlusion**

There was no significant difference in EEG intensity between the control and occlusion groups during the baseline period. In both the 20 and 30 min occlusion groups fetal EEG activity rapidly fell at the onset of occlusion and remained significantly depressed compared to controls throughout occlusion (a mean of $3.6\pm0.4$ dB for both occlusion groups vs. $17.1\pm0.9$ dB in controls, $P<0.001$). Nuchal EMG activity ceased only after the third or fourth minute of occlusion, but during this initial period activity was altered. Fewer movements occurred, but compared to the typical frequent 10 to 15 sec bursts of EMG activity in the baseline period (Fig. 2B), there were periodic, two to three second bursts of higher amplitude EMG activity (Fig. 2C).

**Long-term recovery changes in MAP, FHR and FHRV**

In the 20 min group, FHR was minimally suppressed for the first 5 h after the end of occlusion ($P<0.05$ compared to the control group, Fig. 3). Thereafter there was no significant difference between the 20 min and control groups. The 30 min group there was a significant elevation in FHR between 2-3 hour post-occlusion, and a significant reduction in FHR
between 8 and 24 h, maximal at 19 h (180.2±3.1 vs. 194.7±2.5, \(P<0.001\) vs. controls, Fig. 3A). A “picket fence” or “checkmark” pattern was not seen on the continuous FHR record in either group (20).

The time sequence of changes in MAP showed a significant interaction between time and group \((P<0.001)\). There was no significant difference in MAP between the control and the 20 min occlusion group (Fig. 3). In the 30 min occlusion group there was also a tendency to be elevated during the first 24 h, but there were no differences compared to control until 36 h when MAP was significantly lower than the control and 20 min groups until around 60 h (32.7±0.4 vs. 35.1±0.9 mmHg averaged for this period, \(P<0.05\), Fig. 3).

FHRV was suppressed in both groups during the first 2 h post-occlusion \((P<0.001,\) Fig. 3). In the 20 min group FHRV progressively increased and was transiently greater than controls between 6 and 8 h \((P<0.05)\); thereafter there were no significant differences. In the 30 min group FHRV also rose after 2 h and was not significantly different to control between 3 and 5 h, but fell again and was significantly suppressed for the remainder of the experiment \((P<0.001,\) Fig. 3).

Long-term changes in EEG activity and fetal behavior.

EEG activity in both occlusion groups was profoundly suppressed immediately after release of occlusion. In the 20 min group, EEG amplitude gradually returned to control values over the first 5 h \((P<0.001\) vs. control, Fig.4). Thereafter EEG amplitude was not significantly different to control group values. In the 30 min group there was also a rise in EEG amplitude during the early phase of recovery, but this peaked at 4 h post occlusion well below control values. EEG amplitude then fell and remained significantly suppressed compared to the control group for the remainder of the experiment \((P<0.001,\) Fig. 4A).
Nuchal EMG activity showed a complex pattern of recovery in both asphyxia groups. In the 20 min group nuchal activity was suppressed immediately post-occlusion, and remained suppressed for the first 4 h ($P<0.001$, Fig. 4B, 4C). Nuchal activity then increased between 5 and 8 h, was transiently moderately suppressed again between 9 and 16 h before progressively returning to control group values by 21 h, and remained normal for the remainder of the study in all fetuses. In contrast, in the 30 min group nuchal activity was also suppressed immediately post-occlusion but overall remained significantly suppressed compared to the control group throughout the duration of the study ($P<0.001$ vs. control group, Fig. 4B, 4D). However, nuchal activity did show a phasic pattern, with EMG activity increasing in all fetuses during the early phase of recovery, peaking at around 3 h, before decreasing again. In five fetuses nuchal activity then followed a similar pattern to the 20 min group, with a secondary pronounced increase followed by a gradual return to control values by 66 h post-occlusion. In the remaining half of this group no nuchal activity was observed for the remainder of the experiment.

*Epileptiform activity*

The raw EEG signal after reperfusion showed epileptiform activity consisting of frequent high voltage sharp and spike wave transients in the 30 but not the 20 min group (Fig. 5). These transients appeared 35.8±3.6 min after the end of occlusion and peaked between 3 and 4 h post-occlusion. At 3.5 h spikes occurred at a rate of 31.4±2.1/min at an amplitude of 95.0±13.5µV (Fig. 5B). This activity was superimposed on a significantly suppressed background (3.2±1.3µV vs. 38.8±3.5µV in the control group, Figs. 5A, B & C). This fast and sharp wave spike activity was then followed by a burst-suppression pattern with sustained suppression of the background EEG amplitude (6.1±2.1µV) in all fetuses (Fig. 5C). No high amplitude, low frequency stereotypic evolving seizure activity was seen at any time during
recovery. No abnormal EEG waveforms, other seizure, or burst suppression activity was observed in the 20 min group at any time during recovery (Fig. 5D).

**Histopathology**

No neuronal loss was observed in the sham controls in any region. There was no significant neuronal loss or TUNEL positive cells found in the 20 min group; two animals showed a few neurons with ischaemic cell change and TUNEL labeling (<1%) in the striatum in one fetus, and the cerebellum in the other. Small numbers of TUNEL positive cells were seen in the medulla, e.g. $1.2\pm0.6/0.2\text{mm}^2$ positive cells (mean±SEM) in the nucleus ambiguus. In contrast, the 30 min group showed extensive subcortical neuronal loss with patchy necrosis, stromal edema and local cellular reaction in the striatum, thalamus, the cornu ammonis (CA) fields of the dorsal horn of the hippocampus and the medulla (Fig. 6A). There was very little cortical cell loss (<5% in all fetuses), typically just one to two cells showing ischemic cell change in the parasagittal sulci. There was consistent, severe neuronal loss (range 55 to 95%) in the nuclei of the medulla, as indicated both by ischemic cell change with cytoplasmic homogenization and eosinophilia, and nuclear contraction (Fig. 6B, panel C) and by extensive TUNEL labeling of the nuclei (Fig. 6B, panel D). For example, there were $112.6\pm4.8/0.2\text{mm}^2$ TUNEL positive cells in the nucleus ambiguus; similarly, severe neuronal loss was observed in the nucleus tractus solitarius in the 30 min group but not the 20 min group.
DISCUSSION

The present study demonstrates that exposure to severe asphyxia in the near-midgestation fetus is associated with a complex pattern of changes in FHRV, and that this pattern was markedly affected by the development of neural injury. Strikingly, only the 30 min group, which developed extensive subcortical neural injury including necrosis of brainstem structures, demonstrated profound post-asphyxial suppression of FHRV. This suppression persisted for at least 72 h after reperfusion despite recovery of body movements in a subset of this group. In contrast, the 20 min group, which had no brainstem injury, showed progressive complete recovery of FHRV to control group values. These data, however, highlight a significant potential limitation for clinical interpretation of FHRV since both groups showed an initial progressive increase in FHRV in the first four hours of recovery, but this apparent initial recovery in the two groups was mediated by quite different mechanisms. In the 20 min group it reflected the return of normal body movements, whereas in the 30 min group it corresponded with the appearance of frequent epileptiform activity and abnormal tonic nuchal activity.

Further, our data demonstrate that the preterm fetal sheep has a similar pattern of changes in FHRV during a period of asphyxia to that of the near-term fetus (55), despite the relative immaturity of the preterm autonomic nervous system. We have shown that the increase in FHRV at the onset of asphyxia was closely related to abnormal patterns of body movements, and the increase in FHRV towards the end of 30 min of occlusion was associated with severe irregularity of heart beats. These data are of importance to the interpretation of FHRV both during and after an asphyxial insult in the preterm and term fetus.

Under physiological conditions FHRV reflects, in part, the complex interaction of efferent sympathetic and parasympathetic activity (7, 8, 48), which in turn are influenced by input
from the fetal baroreceptors and chemoreceptors (22, 30, 59), and by fetal behavior (9, 32, 36, 56). In the near-term fetus, hypoxia and asphyxia are associated with a transient, but pronounced increase in MMR, followed by a fall (9, 20, 24, 33, 55). This response is in part mediated by a combination of both increased parasympathetic activity, which mediates the initial fall in FHR, and sympathetic neural activity which mediates increased cardiac contractility and peripheral vasoconstriction (8, 14). Carotid sinus nerve denervation alters the autonomic mediation of the cardiovascular responses to moderate hypoxia (14), and reduces but does not abolish the increase in FHRV at the onset of the insult (24), suggesting that additional factors play a role in this initial rise in FHRV.

The current study demonstrates that behavioral changes are important. In contrast to observations to the term fetus where body movements cease abruptly shortly after the start of asphyxia (34), in the preterm fetus body movements continued for up to five minutes. These movements are more abrupt and of higher amplitude than is seen before occlusion. It may be speculated from a teleological perspective that these jerking movements might be a defense response, helping free the cord from occlusion. The different responses compared with near-term likely reflects the greater anaerobic reserves of the preterm fetus which permits this degree of energy expenditure at such a critical time (49). The subsequent reduction in FHRV corresponds to the cessation of activity and may also reflect hypoxic suppression of brainstem activity (41), and changes in parasympathetic activity (21).

This suppression of FHRV by asphyxia was not maintained when cord occlusion was continued to a near-terminal degree. In the final stages of the 30 min insult, fetuses showed a marked increase in long-term heart rate variability (mean minute rate). This observation is in contrast to the continued suppression observed during more moderate insults in near-term fetuses (20, 24), but is consistent with our previous findings in near-term fetuses exposed to
near-terminal brief repeated umbilical cord occlusion (55). Unlike the initial rise in FHRV, this terminal increase is not related to body movements, which remained wholly suppressed, but rather are due to changes in cardiac activity as shown by the ECG record which showed a mixture of asystole interrupted by abnormal FHR accelerations. Thus, we suggest that this response was related to profound myocardial hypoxia leading to abnormal functioning of the atrial pacemaker. Lending support to this concept, as soon as the fetuses had been reperfused after release of cord occlusion, presumably with return of coronary perfusion (23), FHRV became markedly suppressed in both groups.

Long-term suppression of FHRV after severe asphyxia has been proposed to be a clinical marker of severe antenatal neural injury (1, 40, 47), whose clinical interpretation could be confounded with loss of variability due to chronic hypoxia or to fetal compromise in labor. Postnatal studies in infants with congenital brain lesions suggest that chronic loss of heart rate variability is associated with damage to the medulla oblongata and midbrain (53), likely related to the important roles of key nuclei such as the nucleus tractus solitarius and the nucleus ambiguus in cardiac control (12, 13).

The present study is consistent with this proposed localization, with extensive necrosis of subcortical gray matter, with severe damage to the nucleus ambiguus after 30 min of complete umbilical cord occlusion, but not 20 min. This pattern of selective damage in the brainstem and basal ganglia is typical of preterm infants who have been exposed to profound asphyxia (2, 50), and is in contrast with the common patterns of brain injury in term infants (2). The persistent suppression of FHRV in the second and third days after asphyxia in the 30 min group, and the contrasting complete recovery of FHRV in the 20 min group strongly suggest that the primary mechanism of suppression is brainstem injury (38). Although nuchal activity was initially suppressed, its ultimate recovery in half of the 30 min group suggests that
reduced fetal activity was not the major cause of reduced FHRV. Similarly, there was no prolonged acidemia or hypoxia which might cause suppression (33).

In contrast with this clear-cut separation of the two asphyxial groups in the longer term, the present data uniquely demonstrate that the two groups showed a numerically extremely similar initial recovery of FHRV in the first four hours after asphyxia. However, the mechanisms mediating this increase appear to be completely different. In the 30 min group, a constellation of findings suggest that the initial, transient rise in FHRV after occlusion was a non-specific response reflecting the evolution of subcortical injury. This group demonstrated a parallel increase in total EEG activity, body movements and MMR. The continuous EEG recordings confirmed the presence of frequent epileptiform activity during this phase, which was accompanied by body movements. This pattern is highly consistent with the report that loss of FHRV was not seen until seven hours after recovery from a catastrophic perinatal insult that lead to cystic brainstem injury in a preterm infant (38). Near-term fetal lambs who developed severe neural injury after asphyxia, also showed a transient increase in FHRV, however, the FHRV then became suppressed from 12 h despite continuing classical seizure activity; changes in body movements were not reported (20).

This is the first report of epileptiform activity being observed in the preterm sheep fetus at 0.6 gestation and the first to describe the appearance of EEG transients at any fetal age. In the preterm newborn EEG transients are well documented. Their presence tends to be interpreted conservatively given that sharp and fast transients may be seen in asymptomatic newborns with a normal outcome (46, 54). However, several studies have shown that overall the appearance of such activity is strongly associated with a poor outcome in preterm infants (5, 19, 28, 35, 42, 54). Interestingly, whereas in the near-term ovine fetus overt seizures were seen after severe partial asphyxia in association with a “checkmark” pattern on the FHR
record (20), in the present study true, sustained EEG defined seizures (with a stereotypic evolving pattern of epileptiform activity (45)) were never seen. This difference most likely represents the relative immaturity of neural maturation and myelination and the subcortical nature of the injury in the present study (31).

In the 20 min group FHRV also increased rapidly in the early recovery phase. There was a progressive return to normal patterns of EEG activity and of body movements which were quite marked in the first 6 hours of recovery. Transient epileptiform activity was not seen in this group and thus we postulate that the return of FHRV was directly related to the reappearance of fetal behavior. The mechanisms of the transient overshoot in FHRV during this recovery are unknown, but it may at least in part relate to the return of fetal activity. As in the 30 min group there were, however, no changes in circulating blood gases or glucose levels which might account for the changes in FHRV.

In conclusion, despite the known immaturity of many aspects of sympathetic and vagal control of the cardiovascular system, and the absence of organized behavioral state, many aspects of the pattern of changes in FHRV during and after severe asphyxia in the near-midgestation fetal sheep are similar to that of the mature fetus. Examples of this include the transient initial increase in heart rate variability at the start of occlusion (9, 24, 33, 55), and the long-term suppression of variability in fetuses with severe injury (20). We describe for the first time potential mechanisms mediating the early increase, such as abrupt body movements, and the contrasting mechanism of cardiac instability which appears to mediate the terminal increase in FHRV at the end of the 30 min occlusion period. Critically, this paper has demonstrated that in the early recovery period changes in the numerical amplitude of FHRV were unable to distinguish between a damaging and non-damaging insult; this would be a significant limitation for antenatal monitoring. In contrast, protracted suppression of FHRV
was a strong indicator of severe brainstem damage.

Acknowledgements

This study was supported by the Health Research Council of New Zealand, National Institutes of Health grant RO-1 HD32752, the Lottery Health Board of New Zealand, and the Auckland Medical Research Foundation.
Table 1. Fetal arterial pH, blood gas and lactate values 15 min before (control), during (15 or 25 min after the start of occlusion), and after the end of either sham occlusion (Con), 20 or 30 min of umbilical cord occlusion.

<table>
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<th>Control</th>
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<th>1 h post</th>
<th>6 h post</th>
<th>72 h post</th>
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<tr>
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<td>20</td>
<td>44.7±2.2</td>
<td>129.7±13.0§</td>
<td>43.7±1.8</td>
<td>46.1±0.8</td>
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<td></td>
<td>30</td>
<td>46.0±1.1</td>
<td>154.0±4.4§</td>
<td>43.2±1.3</td>
<td>44.7±0.6</td>
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<td><strong>PaO₂</strong></td>
<td>Con</td>
<td>23.1±0.7</td>
<td>23.5±0.8</td>
<td>23.5±0.6</td>
<td>22.8±0.5</td>
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<tr>
<td>(mmHg)</td>
<td>20</td>
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<td>9.4±0.7§</td>
<td>26.2±0.9*</td>
<td>25.0±0.9</td>
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<tr>
<td></td>
<td>30</td>
<td>24.9±0.9</td>
<td>9.2±0.9§</td>
<td>26.1±1.6</td>
<td>26.7±1.2*</td>
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<td><strong>SaO₂</strong></td>
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<td>71.4±2.3</td>
<td>72.0±2.1</td>
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<tr>
<td>(%)</td>
<td>20</td>
<td>72.0±2.2</td>
<td>8.1±1.0§</td>
<td>76.0±1.5</td>
<td>74.0±3.1</td>
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<td></td>
<td>30</td>
<td>73.2±2.6</td>
<td>6.0±1.1§</td>
<td>78.0±2.9</td>
<td>74.2±2.3</td>
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<tr>
<td><strong>BE</strong></td>
<td>Con</td>
<td>1.4±0.5</td>
<td>2.1±0.3</td>
<td>1.5±0.5</td>
<td>2.3±0.5</td>
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<td>(mmol/L)</td>
<td>20</td>
<td>2.0±1.0</td>
<td>-13.5±2.5§</td>
<td>0.3±1.2*</td>
<td>1.8±0.8</td>
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<tr>
<td></td>
<td>30</td>
<td>1.4±0.5</td>
<td>-15.2±0.9§</td>
<td>-3.0±0.9§</td>
<td>-0.1±0.8*</td>
</tr>
<tr>
<td><strong>Lactate</strong></td>
<td>Con</td>
<td>0.96±0.1</td>
<td>1.02±0.1</td>
<td>1.07±0.1</td>
<td>1.15±0.1</td>
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* indicates significantly different from Con (p<0.05).
§ indicates significantly different from Con (p<0.001).
<table>
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<th>(mmol/l)</th>
<th>20</th>
<th>30</th>
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<td>0.73±0.1</td>
<td>0.84±0.1</td>
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<td>1.70±0.2*</td>
<td>3.34±0.2§</td>
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<tr>
<td></td>
<td>0.90±0.1</td>
<td>1.10±0.4</td>
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Data are mean ± SEM. *P<0.05, ‡ P<0.005, § P<0.001 (control vs. 20 min or 30 min groups by ANCOVA, n=10 in control and 30 min groups, n=7, in the 20 min group). Blood gases at 24 and 48 h were not different from control (data not shown).
Figure Legends

Figure 1. Time sequence of changes in fetal heart rate (FHR), mean arterial blood pressure (MAP), and fetal heart rate variability expressed as mean minute rate (MMR) in the 20 min umbilical cord occlusion group (open squares, n=7) and 30 min group (filled circles, n=10) during and after occlusion. Data are one min averages, mean±SEM. The shaded area highlights the period of occlusion. For clarity the statistics for the 30 min group only are shown. Note that in the 20 min group the data after reperfusion have been plotted starting at 31 min to allow direct comparison with the 30 min occlusion group. *P<0.05, §P<0.001, 30 min group vs. control group, ANOVA.

Figure 2. Panel A shows a 20 second ECG recording from a fetus in the 30 min group, taken from the last 2 min of occlusion. Note the mixture of activity present, with periods of bigeminy, runs of fast beats and asystole. Panels B and C show one min segments of raw nuchal EMG activity, taken just prior to occlusion (baseline activity, panel B) and at 3 min of occlusion (panel C).

Figure 3. Time sequence of changes in fetal heart rate (FHR, beats per min), mean arterial blood pressure (MAP), and fetal heart rate variability measured as mean minute range (MMR) in the control group (open circles), 20 min occlusion group (filled squares), and 30 min occlusion group (filled circles) before and after occlusion (occlusion denoted by an arrow, for data see figure one). Data are mean±SEM averaged over 6 h periods in the 12 h before asphyxia, hourly during the first 24 h after asphyxia and over 6 h periods thereafter. *P<0.05, §P<0.001 vs. control.

Figure 4. Time sequence of changes in fetal electroencephalographic activity (EEG, panel A) and nuchal electromyographic activity (Nuchal EMG, panel B) before and after occlusion in
the control group (open circles), 20 min group (filled squares) and 30 min group (filled circles). Data are mean±SEM averaged over 6 h periods in the 12 h before asphyxia, hourly during the first 24 h after asphyxia and over 6 h periods thereafter. The arrow denotes the period of occlusion, §P<0.001. Panels C and D show continuous one min data from individual fetuses from 12 h before occlusion until 24 h after either 20 min (panel C) or 30 min (panel D) occlusion. The shaded box denotes the period of occlusion.

**Figure 5.** Raw electroencephalographic (EEG) data from one 30 min group fetus showing normal discontinuous mixed frequency EEG activity (panel A), epileptiform transients (fast spikes) superimposed on a suppressed EEG background at 3 h post-occlusion (panel B), and a burst suppression pattern with suppressed background activity at 48 h (panel C). Panel D shows raw EEG data from one 20 min group fetus 3 h post-occlusion showing a mildly reduced amplitude signal but no transients.

**Figure 6.** Figure 6A shows the neuronal loss scores in selected brain regions from the 30 min group, showing severe subcortical damage, in the basal ganglia, hippocampus and brainstem. There was no neuronal loss in sham controls and only trivial cell loss in two of the 20 min group fetuses (<1% neuronal loss scored in the striatum and cerebellum). Data are mean±SEM. Parasag cortex: Parasagittal cortex; DG: dentate gyrus. CA: cornu ammonis of the hippocampus.

Figure 6B shows photomicrographs of the medulla taken at the level of the decussation of the medial lemniscus and the pyramids (the level of the nucleus ambiguus) (15). Panels A to C are acid fuschin/ thionin stained and panel D shows TUNEL staining counterstained with thionin. Panel (A). A sham control fetus showing normal neuronal thionin staining (purple) and appearance. There is no neuronal loss. Panel (B). A fetus exposed to 20 min of asphyxia
also showing normal neurons and no neuronal loss. Panel (C). A fetus exposed to 30 min of asphyxia showing extensive neuronal injury in the brain stem nuclei with severe stromal edema. The arrows indicate examples of dead cells exhibiting ischemic cell change with nuclear condensation and acid fuschin (pink) staining of the cytoplasm. Panel (D). TUNEL staining (brown) from the same fetus, showing extensive DNA fragmentation consistent with the severe neuronal loss in panel C.
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Figure 1
Figure 2.
Figure 3.
Figure 4.
Figure 5
Figure 6a

![Bar graph showing neuronal loss score for different brain regions]

Figure 6b

![Image showing histological sections labeled A, B, C, D with arrows indicating specific features]