Maturation of the Mitochondrial Redox Response to Profound Asphyxia in Fetal Sheep

Paul P. Drury¹, Laura Bennet¹, Lindsea C. Booth¹,², Joanne O. Davidson¹, Guido Wassink¹, Alistair J. Gunn¹,³*

¹Fetal Physiology and Neuroscience Group, Department of Physiology, The University of Auckland, Auckland, New Zealand, ²Howard Florey Institute, University of Melbourne, Melbourne, Victoria, Australia, ³Starship Children’s Hospital, Auckland, New Zealand

Abstract

Fetal susceptibility to hypoxic brain injury increases over the last third of gestation. This study examined the hypothesis that this is associated with impaired mitochondrial adaptation, as measured by more rapid oxidation of cytochrome oxidase (CytOx) during profound asphyxia. Methods: Chronically instrumented fetal sheep at 0.6, 0.7, and 0.85 gestation were subjected to either 30 min (0.6 gestational age (ga), n = 6), 25 min (0.7 ga, n = 27) or 15 min (0.85 ga, n = 17) of complete umbilical cord occlusion. Fetal EEG, cerebral impedance (to measure brain swelling) and near-infrared spectroscopy-derived intra-cerebral oxygenation (ΔHb = HbO2 – Hb), total hemoglobin (THb) and CytOx redox state were monitored continuously. Occlusion was associated with profound, rapid fall in ΔHb in all groups to a plateau from 6 min, greatest at 0.85 ga compared to 0.6 and 0.7 ga (p < 0.05). THb initially increased at all ages, with the greatest rise at 0.85 ga (p < 0.05), followed by a progressive fall from 7 min in all groups. CytOx initially increased in all groups with the greatest rise at 0.85 ga (p < 0.05), followed by a further, delayed increase in preterm fetuses, but a striking fall in the 0.85 group after 6 min of occlusion.

Cerebral impedance (a measure of cytotoxic edema) increased earlier and more rapidly with greater gestation. In conclusion, the more rapid rise in CytOx and cortical impedance during profound asphyxia with greater maturation is consistent with increasing dependence on oxidative metabolism leading to earlier onset of neural energy failure before the onset of systemic hypotension.

Introduction

The mammalian fetus has a remarkable ability to adapt to and survive far more prolonged periods of asphyxia than adults. During fetal life, cardiovascular tolerance to severe asphyxia is typically maximal near-midgestation, corresponding with the time of maximal levels of cardiac glycogen [1], and falls progressively towards term [2]. Neurological tolerance appears to broadly parallel cardiac survival. For example, near-term fetal sheep develop selective neural injury after 10 min of complete umbilical cord occlusion [3,4], with much greater injury and reduced survival with longer insults of up to 15 min [5,6]. In contrast, 0.6 gestation fetal sheep develop little or no injury even after 20 min of occlusion [3,7] and severe, subcortical neural injury requires 30 min of complete occlusion [8].

Potentially then, greater susceptibility to asphyxial neural injury with increasing gestation could be related to either a change in the intrinsic tolerance of the developing brain or to the more rapid onset of profound hypotension and hypoperfusion near-term [2]. In the fetal sheep basal cerebral blood flow and oxygen consumption per 100 g weight increase towards term [9,10], consistent with greater basal neural aerobic dependence that would increase the vulnerability of the brain to asphyxia in late gestation. A similar increase in oxygen consumption and oxygen delivery with increasing gestation is seen in the guinea pig [11], and there is evidence that the preterm brain generates a greater proportion of ATP through non-oxidative metabolism compared to at term [12,13]. Further, in human infants the ratio of phosphocreatine (PCr) to inorganic orthophosphate increases between 28 and 42 weeks gestation, suggesting increasing basal metabolism [14].

More important, effective adaptation to asphyxia must involve the maximum possible reduction in non-essential energy-utilizing processes, particularly synaptic transmission [15–17]. This initial suppression in brain activity is actively mediated by increased levels of inhibitory neuromodulators including adenosine [17,18], which has been termed ‘adaptive hypometabolism’ [19]. There is limited information on how the ability to suppress brain metabolism changes with advancing gestation, however, the minimum energy requirements for essential cell survival seem to be lower earlier in gestation [13,20], likely in part related to reduced dendritic complexity [21]. If this hypothesis is correct, then we may predict that high energy metabolites would be depleted more rapidly during asphyxia closer to term.

These changes can be indirectly monitored by using near-infrared spectroscopy (NIRS) to continuously measure changes in the oxidized state of the CuA moiety of cytochrome oxidase...
Maturation of Fetal Tolerance to Asphyxia

(CytOx) [22]. CytOx is linked to complex IV as the terminal electron acceptor in the electron transport chain. When CytOx has electrons available to be donated it is reduced. Conversely, when all the electrons have been donated to oxygen to form water with H+, creating the transmembrane gradient that drives ATP production, CytOx is oxidized [23]. What determines its redox state then is the balance between electrons being passed down the electron chain and the rate at which they are donated to oxygen. Intracellular ATP levels are buffered by PCr [23], but this pool is very rapidly depleted. Thus, a relative increase in oxidized CytOx suggests either more rapid consumption of electrons to produce ATP (as seen in skeletal muscle during intense exercise [24]), or reduced availability of reducing equivalents from the electron transport chain [25, 26].

In the context of the very rapid, profound de-oxygenation during complete umbilical cord occlusion, an increase in oxidized CytOx must reflect a marked depletion of reducing equivalents transferred from the tricarboxylic acid cycle, rather than increased donation of electrons to oxygen. Consistent with this, we have previously shown that during asphyxia moderately preterm (0.7 gestation) fetal sheep show a rapid initial increase in oxidized CytOx measured using NIRS [27]. Strikingly, there was a substantial delay before CytOx reached peak values, raising the possibility that adaptive hypometabolism may be more effective in preventing injury in the preterm brain than at term, however, this has not been directly assessed.

In the present study we tested the hypothesis that profound asphyxia would be associated with a more rapid initial increase in oxidation of CytOx with greater maturation, before the onset of systemic hypotension. Changes in mitochondrial redox state, cerebral oxygenation, EEG power, cortical impedance (a measure of cell swelling [28]) and carotid blood flow (as an index of global cephalic perfusion [17, 29–32]) were measured during complete umbilical cord occlusion at 0.6, 0.7, and 0.85 gestation. These ages are broadly equivalent to the neural maturation of the human fetus at 26–28 wk, 28–32 wk, and 40 wk gestation, respectively [33, 34]. In separate groups we also assessed changes in local cortical blood flow at 0.7, and 0.83 gestation using laser Doppler [17].

Methods

Surgical procedures

All procedures were approved by the Animal Ethics Committee of the University of Auckland. 38 singleton Romney/Suffolk fetal sheep were operated on at 84–86 d (0.6 of gestation, n = 6), 96–99 d (0.7 of gestation, n = 22 with NIRS optodes plus n = 5 with laser Doppler probes, as below) and 118–125 d (0.85 of gestation, n = 9 with NIRS optodes plus n = 8 with laser Doppler probes) gestational age (term = 147 days). Food, but not water was withdrawn 18 h before surgery. Ewes were given 5 ml of procaine penicillin (250 000 IU) with dihydrostreptomycin (250 mg ml–1, Stockguard Laboratories Ltd, Hamilton, New Zealand) intramuscularly for prophylaxis 30 min prior to the start of surgery. Anesthesia was induced by i.v. injection of Alfaxan (3 mg kg–1; Alphaxalone, Jurox, Rutherford, NSW, Australia), and general anesthesia maintained using 2–3% isoflurane in O2. Under anesthesia a 20-g 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. Ewes were ventilated if necessary and the depth of anesthesia, maternal heart rate and respiration were constantly monitored by trained anesthetic staff.

All surgical procedures were performed using sterile techniques [30]. Following a maternal midline abdominal incision and exteriorization of the uterus and either the top or bottom half of the fetus, catheters were placed in the left fetal femoral artery and vein, right brachial artery and vein, and the amniotic sac. An ultrasonic blood flow probe (size 3S; Transonic Systems Inc., Ithaca, NY, USA) was placed around the left carotid artery to measure carotid artery blood flow (CaBF) as an index of global cephalic blood flow. Two pairs of EEG electrodes (AS633-SSSF, Cooner Wire Co., Chatsworth, CA, USA) were placed through burr holes 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. To measure cortical impedance a third pair of electrodes was placed over the dura 5 mm lateral to the EEG electrodes. A reference electrode was sewn over the occiput. A pair of electrodes were sewn over the fetal chest to measure the fetal ECG. An inflatable silicone occluder was placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, CA, USA). A flexible fiber optic probe (diameter ~400 μm) containing emitting and receiving laser Doppler channels was placed in the right parietal cortex approximately 5 mm lateral to the midline and 15 mm anterior to bregma, to a depth of 5 mm below the dura, in the grey matter of the cortex of 0.7 and 0.85 gestation fetuses only (Oxford Optronix Inc., Oxford, UK) [17]. Two small flexible fiber optic probes, used for the near infrared spectroscopy recordings, were placed biparietally on the skull 3.0 to 3.5 cm apart, 1.5 cm anterior to bregma, and secured using rapid setting dental cement (Rocket Red, Dental Adventures of America, Inc., Anaheim, CA, USA) [27, 35]. NIRS and laser Doppler were recorded in separate groups of animals as light from the laser Doppler interferes with NIRS measurements. All fetal leads were exterterized through the maternal flank and a maternal long saphenous vein was catheterized to provide access for post-operative care and euthanasia. Antibiotics (80 mg Gentamicin, Roussel, Auckland, New Zealand) were administered into the amniotic sac prior to closure of the uterus.

Post-operatively 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 h light/dark cycle. A period of 5 days post-operative recovery was allowed before experiments commenced, during which time antibiotics were administered to the ewe i.v. (4 days 600 mg Benzylpenicillin Sodium; Novartis Ltd, Auckland, New Zealand, and 2 days 80 mg Gentamicin). Fetal catheters were maintained patent by continuous infusion of heparinized saline (20 U/ml at 0.2 ml/h) and the maternal catheter maintained by daily flushing.

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) [36], EEG, CaBF, and impedance were recorded continuously. The blood pressure signal was collected at 64 Hz and low pass filtered at 30 Hz. The EEG signal was high-pass filtered at 1.6 Hz and low-pass filtered at 50 Hz, then stored for offline analysis at a sampling rate of 256 Hz. The high-pass filter had a first-order roll-off of 6 dB per octave, thus attenuating but not removing frequencies below this. Total EEG power (μV2) was calculated from the intensity spectra by fast Fourier transform of the EEG on sequential epochs, using a 10 second Hanning-window to minimize spectral leakage [37]. Cerebral impedance was calculated as previously described [28]. The impedance of a tissue rises concomitantly as cells depolarize and fluid shifts from the extracellular to intracellular space, and thus impedance is a measure of cytotoxic edema. Data were collected by computer and stored to disk for off-line analysis.
Experimental protocol

Experiments were conducted at 88–90 d (0.6), 101–104 d (0.7), and 121–128 d (0.85) gestation. Fetal asphyxia was induced by rapid inflation of the umbilical cord occluder for 30 min in the 0.6 group, 25 min in the 0.7 group and 12–15 min in the 0.85 group with sterile saline of a defined volume known to completely inflate the occluder and totally compress the umbilical cord, as determined in pilot experiments with a Transonic flow probe placed around an umbilical vein [30]. Successful occlusion was confirmed by observation of a rapid onset of bradycardia with a rise in MAP, and by pH and blood gas measurements. If fetal blood pressure fell below 8 mmHg in the 0.6 and 0.7 groups or below 12 mmHg in the 0.85 group then the occlusion was ended immediately. The duration of occlusions were chosen to represent acute, severe, near-terminal insults, associated with severe neuronal loss. All occlusions were undertaken between 0900 and 1000 h. After release of the umbilical cord occluder fetuses were allowed to auto-resuscitate. If fetal heart rate (FHR) was not above 100 bpm within 1 min of occlusion release then 0.1–0.3 ml/kg of 1/10000 adrenaline (DNL, Hospira, Auckland, New Zealand) was administered via the brachial vein. If no response was observed then the ewe was euthanized following fetal death.

Fetal arterial blood was taken at 15 min prior to asphyxia (baseline) and at appropriate early and late time points during asphyxia: 5 and 25 min in the 0.6 group, 5 and 17 min in the 0.7 group, and 2 and 12 min in the 0.85 group during asphyxia 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). At the end of the experiment ewes and fetuses were killed by an intravenous overdose of pentobarbitone sodium (9 g) to the ewe (Pentobarb 300; Chemstock International, Christchurch, New Zealand).

Near-infrared spectroscopy (NIRS) measurements

Concentration changes in fetal cerebral deoxyhemoglobin ([Hb]), oxyhemoglobin ([HbO2]) and oxidised cytochrome oxidase (CytOx) were measured using a NIRS-500 spectrophotometer (Hamamatsu Photonics KK, Hamamatsu City, Japan) and data recorded by computer for off-line analysis. As described previously [38], near-infrared light, at four different wavelengths between 775 and 908 nm, was carried to the fetal head through a fiber optic bundle. Emerging light was collected by the second optode and transmitted to the spectrophotometer. Changes in the cerebral [HbO2], [Hb] and [CytOx] were calculated from the modified Lambert-Beer law using a previously established algorithm which describes optical absorption in a highly scattering medium [39]. These NIRS measures are expressed as relative change from zero. Standardization of the distance between the optodes and fixation of the optodes to the surface of the skull by dental cement were used to reduce signal variability within and between subjects in this study.

Two key parameters were calculated: total hemoglobin ([THb]): the sum of [HbO2] and [Hb], and [ΔHb]: the difference between [HbO2] and [Hb]. THb is related to cerebral blood volume (CBV) by the cerebral hematocrit: 

\[ \text{CBV} = \frac{\text{THb}}{\text{HR}} \]

where H is the arterial hematocrit and R is the cerebral-to-large vessel hematocrit ratio, assumed to be 0.69 [39]. THb is a reliable index of the hemoglobin content of the brain, and thus of CBV, given stable blood hemoglobin and hematocrit [40]. AHi is a measure of total intravascular oxygenation in the brain [41].

Data analysis

Off-line analysis of the physiological data was performed using customized Labview programs. Data were analyzed using JMP 8.0 (SAS Institute, Cary, North Carolina, USA) and SPSS for windows (SPSS, Chicago, II, USA). For between group comparisons analysis of variance for repeated measures was performed. When statistical significance was found one-way analysis of variance with post-hoc LSD tests was used to compare selected time points. Within subjects regression was performed to compare laser Doppler cortical blood flow and carotid artery blood flow using the Bland-Altman method [42]. Statistical significance was accepted when \( p < 0.05 \). Data are mean±SEM.

Results

Umbilical cord occlusion was associated with a progressive, profound hypoxemia, hypercapnia and acidosis; hemoconcentration developed at all ages (Table 1).

Blood pressure, heart rate, carotid blood flow and cortical blood flow

Occlusion was associated with an initial increase in MAP, followed by a rapid fall below baseline values and ultimately with profound hypotension at all ages. MAP was significantly higher in the 0.85 group at baseline (43.3±1.3 vs. 35.6±0.6 and 36.4±0.6 mmHg in the 0.6 and 0.7 ga groups respectively, \( p < 0.05 \)) and for the first 6 minutes of occlusion compared to 0.6 and 0.7 ga fetuses (\( p < 0.05 \)). MAP was also significantly higher over the first 2–7 min in the 0.7 ga compared to the 0.6 ga group (\( p < 0.05 \)). The onset of hypotension occurred earlier with increasing gestation (Figure 1). MAP fell significantly below baseline at 8 min the 0.85 ga group, and 9 min in the 0.6 and 0.7 ga groups (178±5 bpm vs. 191±3 bpm in both 0.6 and 0.7 ga groups, \( p < 0.05 \)). Occlusion was associated with rapid bradycardia followed by a similar gradual further fall in all groups.

CaBF did not change significantly overall in the first 6 min after the onset of occlusion in any group, followed by a progressive fall after the onset of arterial hypotension. There were no differences in CaBF between the 0.6 and 0.7 ga groups. CaBF in 0.85 ga fetuses was significantly lower compared to 0.6 and 0.7 ga from 7–15 min (\( p < 0.05 \)). Cortical laser Doppler flow in 0.85 and 0.7 ga fetuses was highly correlated with CaBF (within subjects regression \( R^2 = 0.62 \), \( p < 0.0001 \), and overall showed a highly similar pattern. However, in contrast with CaBF, over the first 2 min there was a modest increase in laser Doppler flow (\( p < 0.05 \), maximal at 4 min, with no independent effect of gestational age), which resolved to baseline values after 6 min. This was followed by a progressive fall after the onset of hypotension, which was more rapid at 0.85 than 0.7 ga.

EEG and impedance

EEG power was significantly higher at baseline in the 0.85 group compared to the 0.7 and 0.6 ga groups (20.4±0.5 vs. 15.9±0.5 and 14.5±0.9 dB respectively, \( p < 0.05 \), Figure 2). Occlusion was associated with rapid suppression of EEG activity in all groups, with the greatest fall in the 0.85 ga group (EEG power at 2 min of occlusion: 2.1±0.8 dB vs 5.4±1.0 dB in the 0.7 ga and 5.6±1.5 dB in the 0.6 ga groups, \( p < 0.05 \)). There were no significant differences between the 0.6 and 0.7 ga groups. Spectral edge was significantly lower at baseline in the 0.6 ga compared to the 0.7 ga and 0.85 ga groups (7.1±0.7 Hz vs. 10.1±0.4 Hz and 10.4±0.4 Hz respectively, \( p < 0.05 \)). All groups
Table 1. Blood gases, acid-base status, glucose and lactate were measured on fetal arterial blood taken at 15 min prior to asphyxia (baseline) and at an early and late time points during asphyxia: 5 and 25 min at 0.6 gestation, 5 and 17 min at 0.7 gestation, and 2 and 12 min at 0.85 gestation during asphyxia. Hb: hemoglobin concentration; Hct: hematocrit; O2ct: oxygen concentration; BE: base excess.

|               | Baseline | 2/5 min | 12/17/25 min |
|---------------|----------|---------|--------------|
| pH            | 0.6      | 7.38±0.01  | 7.06±0.01     |
|               | 0.7      | 7.36±0.00  | 7.05±0.01     |
|               | 0.85     | 7.39±0.01  | 7.24±0.02     |
| PaCO₂ (mmHg)  | 0.6      | 45.0±0.7   | 89.5±2.8      |
|               | 0.7      | 49.1±0.9   | 99.7±2.8      |
|               | 0.85     | 51.7±1.0   | 68.1±2.8      |
| PaO₂ (mmHg)   | 0.6      | 24.2±0.6   | 6.6±0.9       |
|               | 0.7      | 22.7±0.8   | 5.9±0.5       |
|               | 0.85     | 20.3±0.6   | 6.6±0.6       |
| Hb (g.dL⁻¹)   | 0.6      | 8.2±0.2    | 8.9±0.3       |
|               | 0.7      | 8.6±0.2    | 9.5±0.3       |
|               | 0.85     | 9.9±0.4    | 10.5±0.6      |
| Hct (g.dL⁻¹)  | 0.6      | 23.9±0.6   | 26.0±0.8      |
|               | 0.7      | 25.5±0.6   | 28.2±0.8      |
|               | 0.85     | 29.2±1.3   | 30.8±1.7      |
| O2ct (mmol L⁻¹) | 0.6  | 3.5±0.1    | 0.4±0.1       |
|               | 0.7      | 3.4±0.1    | 0.4±0.0       |
|               | 0.85     | 3.5±0.2    | 0.5±0.0       |
| HCO₃⁻ (mmol L⁻¹) | 0.6  | 25.2±0.5   | 18.1±0.4      |
|               | 0.7      | 25.7±0.4   | 18.6±0.5      |
|               | 0.85     | 28.6±0.4   | 25.0±0.6      |
| BE (mmol L⁻¹) | 0.6      | 1.2±0.5    | -6.2±0.6      |
|               | 0.7      | 2.6±0.4    | -5.5±0.7      |
|               | 0.85     | 4.5±0.5    | 0.3±0.6       |
| Lactate (mmol L⁻¹) | 0.6  | 0.74±0.07  | 3.93±0.21     |
|               | 0.7      | 0.99±0.21  | 3.98±0.15     |
|               | 0.85     | 1.16±0.11  | 2.09±0.17     |
| Glucose (mmol L⁻¹) | 0.6  | 1.11±0.09  | 0.46±0.11     |
|               | 0.7      | 1.06±0.05  | 0.33±0.03     |
|               | 0.85     | 0.86±0.05  | 0.45±0.05     |

*p<0.05 and
**p<0.001 vs. baseline.
†p<0.05 vs. 0.6;
‡p<0.05 and
§p<0.005 vs. 0.85.
doi:10.1371/journal.pone.0039273.t001

showed a rapid suppression of spectral edge frequency with no difference between groups during occlusion. Cortical impedance showed a progressive increase in all groups from several min after the start of occlusion. The relative rise in cortical impedance increased with increasing gestational age; the 0.7 group was significantly greater than the 0.6 group at 9 min (p<0.05) and the 0.85 group was significantly higher than the 0.6 and 0.7 group from 2–15 min (p<0.05). At 10 min of occlusion there was a clear maturation dependent difference in impedance (103±1%, 106±1%, and 129±3%, for 0.6, 0.7, and 0.85 ga respectively, p<0.05). The final maxima, at the end of occlusion, were similar between groups (132±4%, 135±4%, and 138±5%).
Near-infrared spectroscopy

Occlusion was associated with a rapid, profound fall in ΔHb. This fall was greater with increasing gestation (Figure 3), reaching a nadir of 234.0±4.2 µM in the 0.6 group, 242.7±1.7 µM in the 0.7 group, and 247.6±2.4 µM in the 0.85 group (p<0.05). After the nadir there was an apparent increase in ΔHb over the remainder of occlusion in all groups, mediated by a proportionately greater fall in Hb than HbO2; HbO2 did not increase (data not shown). THb initially increased during the compensation phase with a greater rise with increasing gestation, reaching a maxima of 4.7±1.6 µM at 6 min in the 0.6 group, 9.2±0.9 µM at 7 min in the 0.7 group, and 6.0±1.2 µM at 4 min in the 0.85 group (p<0.05, Figure 3). This was followed by a fall in THb in all groups; this fall began earlier in the 0.85 group and was significantly lower than the preterm groups from 6–10 min (p<0.05). Subsequent THb fell in all three groups in parallel, with similar values from around 11 min until the end of their respective occlusion (Figure 3).

Discussion

The present study shows that during profound asphyxia induced by complete umbilical cord occlusion in fetal sheep, greater

![Figure 2. Changes in EEG power, spectral edge frequency, and cortical impedance during occlusion. Data are minute mean±S.E.M. Δ: p<0.05 for 0.85 vs. 0.6 and 0.7 groups; *: p<0.05 for 0.6 vs. 0.7 groups. doi:10.1371/journal.pone.0039273.g002](#)

![Figure 3. Concentration changes in ΔHb (HbO2-Hb), THb (HbO2+Hb) and oxidized cytochrome oxidase (CytOx) during occlusion. Data are one minute mean±S.E.M. Δ: p<0.05 for 0.85 vs. 0.6 and 0.7 groups; o: p<0.05 for 0.6 vs. 0.7 and 0.85 groups; *: p<0.05 for 0.6 vs. 0.7 groups; d: p<0.05 for 0.7 vs. 0.85 groups. doi:10.1371/journal.pone.0039273.g003](#)
maturity was associated with a more rapid rise in oxidized CytOx and greater suppression of cortical EEG activity, followed by markedly more rapid rise in cerebral impedance, indicating more rapid onset of neural depolarization and cell swelling. These findings strongly support the hypothesis that term fetuses are much more dependent on aerobic metabolism during periods of severe hypoxia than earlier in gestation. Unexpectedly, after the initial dramatic increase in oxidized CytOx near-term fetuses then showed a progressive loss of oxidized CytOx to below baseline levels, in contrast with a slow continued rise in the preterm fetuses. The mechanisms of the late fall are unknown, but as reviewed below, this pattern is broadly consistent with reports of a marked fall in oxidized CytOx during hypoxia-ischemia in postnatal animals and during dense cerebral ischemia in the fetal sheep [25,26,43–45]. Thus, speculatively it may reflect either greater cortical injury in near-term fetuses, or maturational changes in the response of the mitochondria.

Cortical impedance rose earlier and much more rapidly near-term than in either the 0.7 or 0.65 gestation preterm fetuses. The impedance of a tissue rises concomitantly as cells depolarize and fluid shifts from the extracellular to the intracellular space causing cell swelling [28], and thus these data indicate earlier onset of neural depolarization and cell swelling with greater maturity. It is striking that impedance increased substantially more in the near-term fetuses. The fall in EEG power in particular was greater and faster in near-term fetuses, and reached a lower absolute nadir at 3 and 4 min of occlusion.

Cortical impedance rose earlier and much more rapidly near-term than in either the 0.7 or 0.65 gestation preterm fetuses. The impedance of a tissue rises concomitantly as cells depolarize and fluid shifts from the extracellular to the intracellular space causing cell swelling [28], and thus these data indicate earlier onset of neural depolarization and cell swelling with greater maturity. It is striking that impedance increased substantially more in the near-term fetuses. The fall in EEG power in particular was greater and faster in near-term fetuses, and reached a lower absolute nadir at 3 and 4 min of occlusion.

Progressive hypotension developed from approximately 9 min-utes of occlusion. The onset of hypotension corresponded closely to the onset of both global and local (cortical) hypoperfusion at all ages. This is highly consistent with the lower limit of cerebral autoregulation being just below baseline blood pressure [59], and with evidence of impaired autoregulation during partial asphyxia in the near-term fetal sheep [60] and inhalational hypoxia in the lamb [61]. In both preterm groups in the present study, the onset of hypotension was associated with a further, delayed rise in oxidized CytOx, followed by linear increases in cortical imped-ance. Similarly, in newborn (postnatal day 7, P7) rats, when brain development is relatively preterm [62], hypoxia-ischemia was associated with an initial reduction of CytOx, followed by delayed oxidation to above baseline levels once ATP levels fell to their nadir [43]. The rises were earlier and more rapid at 0.7 than 0.6 gestation in the present study, despite similar relative falls in blood pressure and carotid blood flow, strongly denoting reduced neural tolerance to anoxia with greater maturity. Supporting this interpretation, the two groups reached essentially identical maxima for oxidized CytOx, suggesting that this late increase in oxidized CytOx reflects progressive loss of production of reducing equivalents due to loss of residual anaerobic metabolism. Poten-tially, active inhibition of mitochondrial function might also contribute to part of the rise, since nitric oxide, for example, is known to inhibit respiratory complexes I and IV [63], and there is evidence that the nitric oxide synthases are more abundant in the immature brain in both sheep and in post-mortem human tissue [64,65].

In contrast, after the onset of hypotension the 0.85 gestation fetuses showed a profound and unexpected fall in oxidized CytOx. This is broadly consistent with the majority of postnatal studies of hypoxia-ischemia, including adults rats [25,26], deep-hypothermic circulatory arrest in the newborn [25] and adult pig [44], and human infants [45], hypotension induced by blood withdrawal during hypoxia in lambs and severe cerebral ischemia in the near-term fetus [66,67]. In contrast, in near-term and newborn lambs moderate hypoxia was associated with an increase in oxidized CytOx [67,68]. It is intriguing to note that in previous studies showing an increase in oxidized CytOx cortical injury was not seen [17,27,67,68]. Conversely, in fetal sheep cerebral ischemia leading to severe cortical injury was associated with a terminal fall in oxidized CytOx, although the precise time course is unknown [69,70].

Despite the apparent fall in measured oxidized CytOx in near-term fetuses, it seems rather improbable that there can been a true shift to more reduced CytOx during profound anoxia, with highly...
limited substrate delivery due to hypotension and hypoglycemia. A more plausible hypothesis is that it reflects a combination of two factors, the increase in brain size with age, and increasing cortical susceptibility to injury. First, in the late gestation fetal sheep brain weight doubles approximately every fortnight. The exact area involved is probably the forebrain, particularly the cerebral cortex, but it is likely that, as the overall size of the brain increases, the cortex would contribute an increasing fraction. Second, a fall in the CytOx signal must reflect a loss of oxidized cytochrome c oxidase; if it is not related to enzymatic reduction, then there could be exposure to the reducing environment of the cytosol either through opening of the mitochondrial permeability transition pore [71], or frank structural damage of the mitochondria. There is evidence for a maturation dependent change in the influence of the mitochondrial permeability transition pore on injury, with apparently little role in immature mice compared to older mice [72]. Further, in vitro, prolonged oxygen-glucose deprivation in rat cortical slices also led to a fall in oxidized CytOx [73], and in blood-free perfused rat brain, cellular ATP only started to fall when CytOx became less oxidized [74]. Thus these data suggest that a fall in oxidized CytOx represents a transition to mitochondrial injury, with apparently little role in immature mice compared to older mice [72].

In conclusion, the present study demonstrates a maturation dependent change in the mitochondrial response to profound asphyxia in fetal sheep, consistent with an intrinsic loss of neural tolerance to severe hypoxia-ischemia. Near-term fetuses showed markedly more rapid increase in oxidation of CytOx, followed by more rapid onset of cytotoxic edema, well before the onset of systemic hypotension or hypoperfusion. During the subsequent progressive cardiovascular decompensation, near-term fetuses showed a more rapid fall blood pressure and carotid and cortical blood flow than preterm fetuses, with accelerated cytotoxic edema, but a paradoxical loss of oxidized cytochrome oxidase. This pattern strongly supports the hypothesis of increasing dependence on aerobic metabolism towards term, independent of the loss of cardiac tolerance to anoxia.

**Author Contributions**

Conceived and designed the experiments: PPD LB AJG. Performed the experiments: PPD LB LCB JOD GW AJG. Analyzed the data: PPD AJG. Wrote the paper: PPD LB LCB JOD GW AJG.

**References**

1. Dawes GS, Mott JC, Shelley HJ (1959) The importance of cardiac glycogen for the maintenance of life in fetal lambs and newborn animals during anoxia. J Physiol 146: 516–538.

2. Wassink G, Bennett L, Booth LC, Jensen EC, Wibbens B, et al. (2007) The ontogeny of hemodynamic responses to prolonged umbilical cord occlusion in fetal sheep. J Appl Physiol 103: 1311–1317.

3. Mallard EC, Williams CE, Johnston BM, Gluckman PD (1994) Increased vulnerability to neuronal damage after umbilical cord occlusion in fetal sheep with advancing gestation. Am J Obstet Gynecol 170: 206–214.

4. Mallard EC, Gunn AJ, Williams CE, Johnston BM, Gluckman PD (1992) Transient umbilical cord occlusion causes hippocampal damage in the fetal sheep. Am J Obstet Gynecol 167: 1423–1430.

5. Ley D, Oksarson G, Bellander M, Hernandez-Andrade E, Langman G, et al. (2004) Different responses of myocardial and cerebral blood flow to cord occlusion in exteriorized fetal sheep. Pediat Res 55: 568–575.

6. Wibbens B, Westgate JA, Bennett L, Roelfsema V, de Haan HH, et al. (2005) Profound hypotension and associated EEG changes during prolonged cord occlusion in the near term fetal sheep. Am J Obstet Gynecol 193: 803–810.

7. Keunable H, Blanco CE, van Reempts JL, Hasaart TH (1997) Absence of neuronal damage after umbilical cord occlusion of 10, 15, and 20 minutes in midgestation fetal sheep. Am J Obstet Gynecol 176: 515–520.

8. George S, Gunn AJ, Westgate JA, Brahyn C, Guan J, et al. (2004) Fetal heart rate variability and brainstem injury after asphyxia in preterm fetal sheep. Am J Physiol Regul Integr Comp Physiol 287: R925–R933.

9. Gleason CA, Hamm C, Jones MD Jr (1989) Cerebral blood flow, oxygenation, and cerebral metabolism in immature fetal sheep in utero. Am J Physiol 256: R1264–R1268.

10. Jensen A, Berger R (1991) Fetal circulatory responses to oxygen lack. J Dev Physiol 16: 181–207.

11. Berger R, Jensen A, Krieglstein J, Steigelmann JP (1991) Cerebral energy metabolism and cerebral metabolism in guinea pig fetuses during development. J Dev Physiol 16: 317–319.

12. Dufy TE, Kohl SJ, Vaccarino RC (1975) Carbohydrate and energy metabolism in perinatal rat brain: relation to survival in anoxia. J Neurochem 24: 271–276.

13. Sylven AL, Sedler EF, Slotkin TA (1989) Effect of transient hypoxia on oxygenation of the developing rat brain: relationships among hemoglobin saturation, autoregulation of blood flow and mitochondrial redox state. J Dev Physiol 12: 287–292.

14. Azzopardi D, Wyatt JS, Hamilton PA, Cady EB, Delphy DT, et al. (1989) Phosphorous metabolites and intracellular pH in the brains of normal and small for gestational age infants investigated by magnetic resonance spectroscopy. Pediat Res 25: 140–144.

15. Astrup J (1980) Energy-requiring cell functions in the ischemic brain. Their critical supply and possible inhibition in protective therapy. J Neurosurg 56: 492–497.

16. Kanelo M, White S, Homan J, Richardson B (2003) Cerebral blood flow and metabolism in relation to electrophysiological activity with severe umbilical cord occlusion in the near-term ovine fetus. Am J Obstet Gynecol 188: 961–972.

17. Hunter CJ, Bennett L, Power GG, Roelfsema V, Blood AB, et al. (2005) Key neuroprotective role for endogenous adenosine A1 receptor activation during asphyxia in the fetal sheep. Stroke 36: 2239–2245.

18. Bie A, Ciocon D, Zagpane AM, Nita DA, Zagpane L, et al. (2006) Endogenous activation of adenosine A1 receptors accelerates ischemic suppression of spontaneous electrophysiological activity. J Neurophysiol 96: 2809–2814.

19. Mortola JP (2004) Implications of hypoxic hypermetabolism during mammalian ontogenesis. Respir Physiol Neurobiol 141: 345–356.

20. Guan AJ, Qiadackers JS, Guan J, Heineman E, Bennett L (2001) The premature fetus: not as defenseless as we thought, but still paradoxically vulnerable? Dev Neurosci 23: 175–179.

21. Dieu S, Rees S (2005) Dendritic morphology is altered in hippocampal neurons following prenatal compromise. J Neurobiol 55: 41–52.

22. Babies RR (1974) Noninvasive, infrared monitoring of cerebral and myocardial oxygen saturation and circulatory parameters. Science 198: 1264–1267.

23. Mozor T, Banaji M, Robertson NJ, Cooper CE, Tachtsidis I (2012) Different fetal responses to asphyxia: a review. Can J Physiol Pharmacol 90: 482–497.

24. Boushel R, Piantadosi CA (2000) Near-infrared spectroscopy for monitoring muscle oxygenation. Acta Physiol Scand 168: 615–622.

25. Sprigget RJ, Wylezinska M, Cady EB, Hollis V, Cope M, et al. (2005) Prenatal oxidative stress and the risk of cerebral palsy: a fetal sheep model. PLoS ONE 10: e13498–e13507.

26. Miyamoto H, Oda T, Tanno IA, Yoshitani N, Tsuchiya N (1996) Does the redox state of cytochrome aa3 reflect brain energy level during hypoxia? Simultaneous measurements by near infrared spectrophotometry and 31P nuclear magnetic resonance spectroscopy. Anesth Analg 83: 513–518.

27. Bennett L, Roelfsema V, Dean J, Wassink G, Power GG, et al. (2007) Regulation of cytochrome oxidase redox state during umbilical cord occlusion in preterm fetal sheep. Am J Physiol Regul Integr Comp Physiol 292: R1569–R1576.

28. Williams CE, Gunn A, Gluckman PD (1991) Time course of intracellular edema and epileptiform activity following prenatal cerebral ischemia in sheep. Stroke 22: 516–521.
29. van Bel F, Roman C, Klautz RJ, Teitel DF, Rudolph AM (1994) Relationship between brain blood flow and carotid arterial flow in the sheep fetus. Pediat Res 35: 329–333.

30. Bennet L, Rossmorele S, Gunning MI, Gluckman PD, Gunn AJ (1999) The cardiovascular and cerebrovascular responses of the immature fetal sheep to acute umbilical cord occlusion. J Physiol 517: 247–257.

31. Gonzalez H, Hunter CJ, Bennet L, Power GG, Gunn AJ (2005) Cerebral oxygenation during post-asphyxial seizures in near-term fetal sheep. J Cereb Blood Flow Metab 25: 911–918.

32. Dummhoor DR, Quilligan EJ (1973) Carotid blood flow distribution in the in utero sheep fetus. American Journal of Obstetrics and Gynecology 116: 648–656.

33. McIntosh GH, Baghurst KL, Potter BJ, Hetzel BS (1979) Foetal brain development in the sheep. Neuropathol Appl Neurobiol 5: 103–114.

34. Barlow RM (1969) The foetal sheep: morphogenesis of the nervous system and histochemical aspects of myelination. J Comp Neuroci 135: 249–262.

35. Bennet L, Roelfsema V, Pathipati P, Quadeckers J, Gunn AJ (2006) Relationship between evolving epileptiform activity and delayed loss of mitochondrial activity after asphyxia measured by near-infrared spectroscopy in preterm fetal sheep. J Physiol 572: 141–154.

36. Lawler FH, Bruce RA (1982) Fetal and maternal arterial pressures and heart rates: histograms, correlations, and rhythms. Am J Physiol 243: R433–R444.

37. Williams CE, Gunn AJ, Mallard C, Gluckman PD (1992) Outcome after ischemia in the developing sheep brain: an electrophenographic and histological study. Ann Neurol 31: 14–21.

38. Reynolds EO, Wyatt JS, Azopardi D, Delpy DT, Candy EB, et al. (1983) New non-invasive methods for assessing brain oxygenation and haemodynamics. Br Med Bull 44: 1052–1073.

39. Wyatt JS, Cope M, Delpy DT, Richardson CE, Edwards AD, et al. (1990) Quantitation of cerebral blood volume in human infants by near-infrared spectroscopy. J Appl Physiol 68: 1086–1091.

40. Barfield CP, Yu YY, Noma O, Kakita J, Cussen LJ, et al. (1999) Cerebral blood volume measured using near-infrared spectroscopy and radionuclide in the immature lamb brain. Pediat Res 46: 36–56.

41. Bruin NC, Moen A, Borch K, Saugstad OD, Greisen G (1997) Near-infrared monitoring of cerebral tissue oxygen saturation and blood volume in newborn piglets. Am J Physiol 273: H602–608.

42. Bland JM, Altman DG (1995) Calculating correlation coefficients with repeated observations: Part 1–Correlation within subjects. Bmj 310: 446.

43. Yager JY, Brucklacher RM, Vannucci RC (1996) Paradoxical mitochondrial activity after asphyxia measured by near-infrared spectroscopy in preterm fetal sheep. J Physiol 572: 141–154.

44. Gagnon RE, Macnab AJ, Gagnon FA, Leblanc JG (2005) Brain, spine, and hemodynamic effects of carotid ligation in lambs. Pediatr Res 34: 51–55.

45. du Plessis AJ, Mooij CA, Hanm C, Jones MD Jr (1996) Effect of acute hypoxemia on brain blood flow and oxygen metabolism in immature fetal sheep. Am J Physiol 258: H1064–H1069.

46. Shadid M, Hiltermann L, Monteiro L, Fontijn J, van Bel F (1999) Near infrared spectroscopy-measured changes in cerebral blood volume and cytochrome aa3 isoforms is reduced in late-gestation ovine fetal brainstem. Am J Physiol Regul Integr Comp Physiol 289: R613–R619.

47. Shadid M, Hiltermann L, Monteiro L, Fontijn J, van Bel F (1999) Near infrared spectroscopy-measured changes in cerebral blood volume and cytochrome aa3 in newborn lambs exposed to hypoxia and hypercapnia, and ischemia: a comparison with changes in brain perfusion and O2 metabolism. Early Hum Dev 55: 169–182.

48. Wood CE, Chen GF, Keller-Wood M (2005) Expression of nitric oxide synthase isoforms is reduced in late-gestation ovine fetal brainstem. Am J Physiol Regul Integr Comp Physiol 289: R613–R619.

49. Johnson GN, Fothergill M, Ware D (1985) Autoregulation of cerebral blood flow during severe fetal asphyxia in late gestation fetal sheep. Pediatr Res 19: 56–61.

50. Romijn HJ, Hofman MA, Grasbergen A (1991) At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby? Early Hum Dev 26: 61–67.

51. Marks KA, Mallard EC, Roberts I, Williams CE, Sirimanne ES, et al. (1996) Delayedvasodilation and altered oxygenation after cerebral ischemia in fetal sheep. Pediatr Res 39: 48–54.

52. Romijn HJ, Hofman MA, Grasbergen A (1991) At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby? Early Hum Dev 26: 61–67.

53. Marks KA, Mallard EC, Roberts I, Williams CE, Sirimanne ES, et al. (1996) Delayedvasodilation and altered oxygenation after cerebral ischemia in fetal sheep. Pediatr Res 39: 48–54.

54. Brown GC (1997) Nitric oxide inhibition of cytochrome oxidase and mitochondrial respiration: implications for inflammatory, neurodegenerative and ischemic pathologies. Mol Cell Biochem 174: 189–192.

55. Jensen A, Hohmann M, Kunzel W (1987) Dynamic changes in organ blood flow during severe fetal asphyxia produced by slow umbilical cord occlusion. J Physiol 517: 247–257.

56. Romijn HJ, Hofman MA, Grassbergen A (1991) At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby? Early Hum Dev 26: 61–67.

57. Barcroft J (1946) Researches in prenatal life. London and Oxford: Blackwell Scientific Publications Ltd.

58. Iskowitz J, Rudolph AM (1982) Denervation of arterial chemoreceptors and baroreceptors in fetal lambs in utero. Am J Physiol 242: H916–H929.

59. Barfield CP, Yu YY, Noma O, Kakita J, Cussen LJ, et al. (1999) Cerebral blood volume measured using near-infrared spectroscopy and radionuclide in the immature lamb brain. Pediatr Res 46: 36–56.

60. Barcroft J (1946) Researches in prenatal life. London and Oxford: Blackwell Scientific Publications Ltd.

61. Barcroft J (1946) Researches in prenatal life. London and Oxford: Blackwell Scientific Publications Ltd.

62. Ball RH, Parer JF, Caldwell LE, Johnson J (1994) Regional blood flow and metabolism in ovine fetuses during severe cord occlusion. Am J Obstet Gynecol 171: 1549–1555.

63. Bennet L, Roelfsema V, Pathipati P, Quadeckers J, Gunn AJ (2006) Relationship between evolving epileptiform activity and delayed loss of mitochondrial activity after asphyxia measured by near-infrared spectroscopy in preterm fetal sheep. J Physiol 572: 141–154.

64. Ball RH, Parer JF, Caldwell LE, Johnson J (1994) Regional blood flow and metabolism in ovine fetuses during severe cord occlusion. Am J Obstet Gynecol 171: 1549–1555.

65. Jensen A, Hohmann M, Kunzel W (1987) Dynamic changes in organ blood flow during severe fetal asphyxia produced by slow umbilical cord occlusion. J Physiol 517: 247–257.

66. Ball RH, Parer JF, Caldwell LE, Johnson J (1994) Regional blood flow and metabolism in ovine fetuses during severe cord occlusion. Am J Obstet Gynecol 171: 1549–1555.

67. Shadid M, Hiltermann L, Monteiro L, Fontijn J, van Bel F (1999) Near infrared spectroscopy-measured changes in cerebral blood volume and cytochrome aa3 in newborn lambs exposed to hypoxia and hypercapnia, and ischemia: a comparison with changes in brain perfusion and O2 metabolism. Early Hum Dev 55: 169–182.

68. Wood CE, Chen GF, Keller-Wood M (2005) Expression of nitric oxide synthase isoforms is reduced in late-gestation ovine fetal brainstem. Am J Physiol Regul Integr Comp Physiol 289: R613–R619.

69. Wood CE, Chen GF, Keller-Wood M (2005) Expression of nitric oxide synthase isoforms is reduced in late-gestation ovine fetal brainstem. Am J Physiol Regul Integr Comp Physiol 289: R613–R619.

70. Wray S, Cope M, Delpy DT, Wyatt JS, Reynolds EO (1988) Characterization of the near infrared absorption spectra of cytochrome aa3 and hemoglobin for the near infrared spectroscopy-measured changes in cerebral blood volume and cytochrome aa3 in perfused rat brain in situ. Am J Physiol 275: C1022–1030.

71. Wray S, Cope M, Delpy DT, Wyatt JS, Reynolds EO (1988) Characterization of the near infrared absorption spectra of cytochrome aa3 and hemoglobin for the near infrared spectroscopy-measured changes in cerebral blood volume and cytochrome aa3 in perfused rat brain in situ. Am J Physiol 275: C1022–1030.

72. Wray S, Cope M, Delpy DT, Wyatt JS, Reynolds EO (1988) Characterization of the near infrared absorption spectra of cytochrome aa3 and hemoglobin for the near infrared spectroscopy-measured changes in cerebral blood volume and cytochrome aa3 in perfused rat brain in situ. Am J Physiol 275: C1022–1030.

73. Wray S, Cope M, Delpy DT, Wyatt JS, Reynolds EO (1988) Characterization of the near infrared absorption spectra of cytochrome aa3 and hemoglobin for the near infrared spectroscopy-measured changes in cerebral blood volume and cytochrome aa3 in perfused rat brain in situ. Am J Physiol 275: C1022–1030.
Author/s:
Drury, PP; Bennet, L; Booth, LC; Davidson, JO; Wassink, G; Gunn, AJ

Title:
Maturation of the Mitochondrial Redox Response to Profound Asphyxia in Fetal Sheep

Date:
2012-06-15

Citation:
Drury, P. P., Bennet, L., Booth, L. C., Davidson, J. O., Wassink, G. & Gunn, A. J. (2012). Maturation of the Mitochondrial Redox Response to Profound Asphyxia in Fetal Sheep. PLOS ONE, 7 (6), https://doi.org/10.1371/journal.pone.0039273.

Persistent Link:
http://hdl.handle.net/11343/264542

File Description:
Published version

License:
CC BY