*Original Article*

JCBFM

Journal of Cerebral Blood Flow & Metabolism
2018, Vol. 38(4) 706–718

DOI: 10.1177/0271678X17703124
journals.sagepub.com/home/jcbfm
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# Antenatal dexamethasone before asphyxia promotes cystic neural injury in preterm fetal sheep by inducing hyperglycemia

**Christopher A Lear, Joanne O Davidson, Georgia R Mackay, Paul P Drury, Robert Galinsky, Josine S Quaedackers, Alistair J Gunn and Laura Bennet**

**Abstract**

Antenatal glucocorticoid therapy significantly improves the short-term systemic outcomes of prematurely born infants, but there is limited information available on their impact on neurodevelopmental outcomes in at-risk preterm babies exposed to perinatal asphyxia. Preterm fetal sheep (0.7 of gestation) were exposed to a maternal injection of 12 mg dexamethasone or saline followed 4 h later by asphyxia induced by 25 min of complete umbilical cord occlusion. In a subsequent study, fetuses received titrated glucose infusions followed 4 h later by asphyxia to examine the hypothesis that hyperglycemia mediated the effects of dexamethasone. Post-mortems were performed 7 days after asphyxia for cerebral histology. Maternal dexamethasone before asphyxia was associated with severe, cystic brain injury compared to diffuse injury after saline injection, with increased numbers of seizures, worse recovery of brain activity, and increased arterial glucose levels before, during, and after asphyxia. Glucose infusions before asphyxia replicated these adverse outcomes, with a strong correlation between greater increases in glucose before asphyxia and greater neural injury. These findings strongly suggest that dexamethasone exposure and hyperglycemia can transform diffuse injury into cystic brain injury after asphyxia in preterm fetal sheep.

**Keywords**

Glucocorticoids, hyperglycemia, asphyxia, premature birth, periventricular leukomalacia

Received 20 January 2017; Revised 4 March 2017; Accepted 13 March 2017

## Introduction

Hypoxic-ischemic encephalopathy (HIE) in premature infants occurs much more frequently than at term,[1] and contributes to the life-long neurodevelopmental disabilities associated with premature birth, including cerebral palsy.[2,3] Mothers at risk of preterm birth are routinely administered synthetic glucocorticoids. Current evidence suggests, reassuringly, that this improves short-term outcomes, with no adverse neurodevelopmental effects for the majority of preterm infants.[4] However, there is a considerable gap in our knowledge of whether antenatal glucocorticoids modulate brain injury in the high-risk subset of preterm infants with HIE.[5,6] Current evidence is largely derived from studies in postnatal day 7 (P7) rats, which consistently report strong neuroprotective effects of dexamethasone when given 4–48 h before hypoxia-ischemia (HI),[7] as reviewed.[6] There is recent evidence however that pretreatment started earlier before HI is deleterious.[8,9] When neuroprotection has been observed in postnatal rat studies, it was, at least in part, mediated by hyperglycemia.[6,10,11]

It is of concern that these findings are inconsistent with some clinical outcomes. The doses and route of administration in postnatal rat studies are more equivalent to clinical postnatal glucocorticoid treatment for

Department of Physiology, The University of Auckland, Auckland, New Zealand

**Corresponding author:**
Laura Bennet, Faculty of Medical and Health Sciences, Department of Physiology, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand.
Email: l.bennet@auckland.ac.nz

chronic lung disease,[12] rather than antenatal treatment. Recent meta-analysis suggests that postnatal glucocorticoid treatment (most commonly dexamethasone) is associated with increased risk of abnormal neurological examination and cerebral palsy despite improved short-term outcomes.[12] The impact of antenatal and postnatal treatment on the brain are likely to be different since antenatal treatment is typically continued for a much shorter duration than postnatal treatment and can involve different glucocorticoids and doses.[4] Moreover, clinically, hyperglycemia is also associated with adverse neurodevelopmental outcomes both in preterm infants,[13,14] and in term infants with HIE.[15,16]

The only large animal study of antenatal glucocorticoid pretreatment, in term-equivalent fetal sheep, suggested that maternal dexamethasone given 48 h before cerebral ischemia did not reduce neural injury.[17] Although there have been no studies in preterm-equivalent large animals, infusion of glucose before HI or ischemia increased brain injury in term neonatal dogs and piglets and near-term fetal sheep.[18–20] We have recently shown that maternal dexamethasone exposure after asphyxia in preterm fetal sheep moderately increased brain injury,[21] in association with altered coupling of cerebral blood flow and metabolism and exaggerated post-asphyxial hyperglycemia.[22]

In this study, we investigated the effects of antenatal dexamethasone on asphyxial brain injury using a clinically relevant dose and route of administration (12 mg maternal intramuscular injection) given 4 h before asphyxia in preterm fetal sheep at 0.7 of gestation, when brain maturity is equivalent to the 28- to 32-week human preterm fetus.[23] We then examined the independent effects of hyperglycemia by giving intravenous glucose infusions from 4 h before asphyxia. In contrast to the findings of postnatal rat studies, dexamethasone treatment and glucose infusions before asphyxia were associated with a consistent transformation of brain injury from diffuse, selective injury to frank cystic infarction.

## Materials and methods

### Ethical approval

All procedures were approved by the Animal Ethics Committee of the University of Auckland, and carried out in accordance with the New Zealand Animal Welfare Act 1999 and the University of Auckland's Code of Ethical Conduct for the use of animals for teaching and research, approved by the Ministry of Primary Industries, Government of New Zealand. This manuscript is compliant with the ARRIVE guidelines for reported animal research.[24]

### Surgical procedures

Forty Romney/Suffolk, fetal sheep were surgically instrumented at 98–100 days of gestation (term is 147 days) as previously described.[22,25,26] Ewes were given long acting oxytetracycline (20 mg/kg, Phoenix Pharm Distributors, Auckland, New Zealand) intramuscularly 30 min before surgery for antibiotic prophylaxis. Anesthesia was induced by intravenous injection of propofol (5 mg/kg, AstraZeneca, Auckland, New Zealand) and general anesthesia was maintained using 2–3% isoflurane in oxygen. The depth of anesthesia, maternal heart rate, and respiration were constantly monitored by trained anesthetic staff. Ewes received a constant infusion of isotonic saline (approximately 250 mL/h) to maintain fluid balance.

A midline abdominal incision was made to expose the uterus, and the fetus was partially exteriorized for instrumentation. Polyvinyl catheters (SteriHealth, Dandenong South, VIC, Australia) were placed in the left femoral artery (with the catheter positioned to sit in the abdominal aorta to measure arterial blood pressure), the left femoral vein (with the catheter positioned to sit in the inferior vena cava to allow intravenous glucose infusions), and the right brachial artery for pre-ductal blood sampling. An additional catheter was placed into the amniotic sac for measurement of pressure within the amniotic space. Electrodes (AS633-5SSF, Cooner Wire, Chatsworth, CA, USA) were placed subcutaneously over the right shoulder and at the level of the left fifth intercostal space to measure the fetal electrocardiogram (ECG). Two pairs of electrodes (Cooner Wire) were placed on the dura over the parasagittal parietal cortex bilaterally, 10 and 15 mm anterior and 5 mm lateral to bregma to measure fetal EEG activity. A pair of electrodes was placed bilaterally on the dura 12.5 mm anterior, 7.5 mm lateral to bregma to measure cortical impedance. Cortical impedance provides a measure of cortical edema and swelling. The impedance of a tissue rises concomitantly as cells depolarize and fluid shifts from the extracellular space to the intracellular space in association with the development of cytotoxic edema.[27] An additional reference electrode was sewn over the occiput. Finally, an inflatable silicone occluder was placed around the umbilical cord to facilitate umbilical cord occlusions (OC16HD, In Vivo Metric, Healdsburg, CA, USA).

Gentamicin was administered into the amniotic sac (80 mg, Pfizer, Auckland, New Zealand) before the uterus was closed. The maternal midline skin incision was infiltrated with a local analgesic, 10 mL 0.5% bupivacaine plus adrenaline (AstraZeneca) to provide long-acting analgesia. All fetal leads were exteriorized through the maternal flank, and a maternal long saphenous vein was catheterized for post-operative care and euthanasia.

### Post-operative care

Following surgery, ewes were housed together in separate metabolic cages with ad libitum access to food and water. Rooms were temperature and humidity controlled ($16 \pm 1^{\circ}C$, humidity $50 \pm 10\%$) with a 12 h light/dark cycle (light 0600 to 1800 h). Ewes were given daily intravenous antibiotics (600 mg benzylpenicillin sodium, Novartis, Auckland, New Zealand, and 80 mg gentamicin) for 4 days after surgery. Fetal catheters were maintained patent with continuous infusion of heparinized saline (20 U/mL at 0.2 mL/h).

### Data acquisition and recordings

Fetal arterial blood pressure, ECG, EEG, and cortical impedance were recorded continuously from 24 h before until 168 h (7 days) after umbilical cord occlusion.[22] All signals were processed and initially digitized at a sampling rate of 4096 Hz before being decimated to lower sampling rates and stored using customized Labview-based data acquisition software (National Instruments, Austin, TX, USA). Fetal arterial blood pressure was recorded using Novatrans III Gold, MX860 pressure transducers (Medex, Hilliard, OH, USA) and corrected for maternal position by subtraction of amniotic fluid pressure. The pressure signals were amplified 500×, low-pass filtered with a Butterworth filter set at 20 Hz and saved at 64 Hz. The raw ECG signal was analogue filtered with a first-order high-pass filter set at 1 Hz and an eighth-order low-pass Bessel filter set at 100 Hz and saved at 1024 Hz. RR intervals were extracted from this signal to calculate fetal heart rate. EEG signals were amplified $10{,}000 \times$ and processed with a sixth-order low-pass Butterworth anti-aliasing filter set to 50 Hz. Total EEG power ($\mu V^2$) was calculated on the power spectrum between 1 Hz and 20 Hz and log transformed for presentation (dB, $20 \times \log$ (power)).[28,29] EEG spectral edge frequency was calculated as the frequency below which 90% of EEG power was present.[29] The raw EEG signal was additionally processed through an Inverse Chebyshev low-pass filter with a cut-off frequency of 512 Hz, and saved at 1024 Hz for analysis of the raw EEG waveforms. Cortical impedance was measured by injecting a sinusoidal current with a frequency of 200 Hz and amplitude of 0.2 μA through the two cortical impedance electrodes. Cortical impedance was calculated by measuring the voltage drop across the front two EEG electrodes.[27]

### Experimental protocol

Experiments began between 09:00 and 09:30 h, 4–6 days after surgery when fetuses were at 104–105 days of gestation. Fetuses were randomly assigned to one of five groups: (a) maternal saline plus sham-asphyxia (saline-sham, n = 8), (b) maternal dexamethasone plus sham-asphyxia (dexamethasone-sham, n = 9), (c) maternal saline plus asphyxia (saline-asphyxia, n = 9), (d) maternal dexamethasone plus asphyxia (dexamethasone-asphyxia, n = 7), and (e) maternal saline plus fetal glucose and asphyxia (glucose-asphyxia, n = 7). Maternal treatment of either dexamethasone (12 mg dexamethasone phosphate, Hameln Pharmaceuticals, Gloucester, UK) or saline was given as a 3 mL intramuscular injection. Fetuses assigned to the glucose-asphyxia group received a continuous intravenous infusion of glucose (Sigma-Aldrich, Sydney, NSW, Australia) dissolved in sterile saline (2 mmol/mL). The glucose infusion rate (average $15.6 \pm 2.2$ mg/kg/min) was titrated over a 4-h period before umbilical cord occlusions to cause a similar increase in arterial blood glucose observed 4 h after maternal dexamethasone injection. Fetal asphyxia was induced 4 h after maternal treatment by complete umbilical cord occlusion for 25 min. Occlusion was ended early if MAP fell below 8 mmHg.[25] Sham-asphyxia fetuses received no occlusion. In the glucose-asphyxia group, the glucose infusion was stopped at the start of umbilical cord occlusion.

### Arterial blood samples

Fetal arterial blood samples (0.3 mL) were taken before the start of the experiment (before maternal treatment), 5 min prior to start of occlusion, at 5 and 17 min during occlusion, and at 10 min, 1, 2, 4, and 6 h after occlusion, then daily thereafter between 08:30 and 09:30 h. Additional arterial blood samples (0.05 mL) were taken in the glucose-asphyxia group over the 4-h infusion period to measure glucose levels and allow the infusion rate to be titrated appropriately. Blood samples were analyzed for pH and blood gases (ABL 800, Radiometer, Copenhagen, Denmark) and glucose and lactate levels (YSI model 2300, Yellow Springs, OH, USA). Seven days after occlusion, ewes and fetuses were humanely killed by an overdose of sodium pentobarbitone given intravenously to the ewe (9 g Pentobarb 300, Provet New Zealand, Auckland, New Zealand).

### Neurophysiological data analysis

Offline analysis of the physiological data was carried out using customized Labview-based software (National Instruments). The three asphyxia groups were analyzed as 1 min means to investigate the neurophysiological adaptation to asphyxia while all five groups were analyzed as 1 h means to investigate the neurophysiological recovery after asphyxia. Spectral edge frequency is displayed as its absolute value,

while both EEG power and cortical impedance were normalized to their baseline values. Baseline was defined as the average of the 20-h period prior to maternal treatment or the start of fetal glucose infusions. EEG power is displayed as change in power (ΔEEG power, dB) while cortical impedance is displayed as percentage of baseline. Additionally, the rate of change of cortical impedance every minute was calculated during the period of asphyxia as the difference between successive minute averages. The cortical impedance signal could not be recorded in one fetus from the saline-asphyxia group and one fetus in the glucose-asphyxia group.

Overt electrographic seizures and seizure-like epileptiform activity were visually identified and assessed from the continuous raw EEG traces in the three asphyxia groups. Seizures were defined as the concurrent appearance of sudden, repetitive, and rhythmic waveforms in the EEG signal lasting $>10$ s[30] with or without a stereotypic evolving nature. In the saline-asphyxia group, all post-asphyxial seizures were counted and assessed for amplitude and duration. In the dexamethasone-asphyxia and glucose-asphyxia groups, all post-asphyxial seizures within 72 h after asphyxia were identified and counted. Due to the high number of seizures in the dexamethasone-asphyxia and glucose-asphyxia groups, analysis of seizure amplitude and duration were performed through batch sampling: all seizures occurring within the final hour of every 6-h period after asphyxia were analyzed for amplitude and duration. Total seizure period was calculated as the time between the first and final seizure-like waveform. The EEG trace was assessed until the end of recovery for the appearance of abnormal patterns, including burst suppression patterns defined as prolonged periods ($>10$ s) of marked EEG suppression ($<5$ mV) interspersed with paroxysm bursts of activity.[31]

## Histological preparation

At post-mortem, fetal brains were perfusion fixed in situ with 10% phosphate-buffered formalin and remained in formalin for 1 week before being processed and paraffin embedded. 10-μm thick coronal sections were cut using a rotary microtome (RM2235, Leica Microsystems, Wetzlar, Germany) and one section from two cerebral regions was selected from each fetus. The first section was taken 27 mm anterior to stereotaxic zero[32] to examine the striatum (comprising the caudate nucleus and putamen). The second section was taken 17 mm anterior to stereotaxic zero[32] to examine the sagittal gyrus, white matter, dorsal horn of the anterior hippocampus and the thalamus. Slides were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol and subsequently washed in 0.1 mol/L phosphate-buffered saline. Slides were stained with thionine (Scharlau, Barcelona, Spain) followed by acid fuchsin (Sigma-Aldrich) to examine overall gross structural integrity.

## Histological analysis

Five regions of interest (striatum, white matter, sagittal gyrus, hippocampus, and thalamus) were examined by light microscopy at 2–10× magnification by an assessor (C.A.L.) blinded to the group using a Nikon eclipse 80i microscope with automated motorized stage (Nikon Corporation, Tokyo, Japan). Using Stereoinvestigator software (Microbrightfield Bioscience, Williston, VT, USA), each region of interest on both hemispheres was separately quantified to measure the total area of the region. The area of white matter quantified included all continuous white matter structures including the intragyral regions, the periventricular and lateral white matter and corpus callosum. The area of the sagittal gyrus included both the cortex and underlying intragyral white matter; the inferior boarder of the gyrus was defined as a straight line bisecting the apex of the cingulate sulcus and the apex of the longitudinal fissure. The area of the hippocampus included the Cornu Ammonis and dentate gyrus and the associated white matter.

The total area of each region was divided into intact tissue and non-intact tissue. Intact tissue was defined as showing intact morphology and structure without macroscopic loss of cellular components, while non-intact tissue was defined as showing irregular macroscopic architecture or cellular loss that was macroscopically evident and consistent with an infarction. Tissue that appeared irregular for the region and consistent with a glial scar was classed as non-intact tissue. The intact area of each region of interest was averaged across both hemispheres to obtain the final intact area for each region. Final images for publication were imaged on a Zeiss Axio Imager Z2 microscope with automated motorized stage (Carl Zeiss AG, Oberkochen, Germany). Serial images were collected at 10× magnification and collated using VSlide stitching software (MetaSystems, Altlussheim, Germany).

## Statistical analysis

Statistical analysis was performed using SPSS v23 (SPSS, Chicago, IL, USA). During analysis of histological outcomes, neurophysiological recovery, and biochemical parameters, we treated data as two separate studies. First, the four core groups (saline-sham, dexamethasone-sham, saline-asphyxia, and dexamethasone-asphyxia) formed a fully cross-matched study. MANOVA was used with dexamethasone and asphyxia

treated as independent factors. For neurophysiological and biochemical parameters, time was treated as a repeated measure while the five brain regions were treated as repeated measures during analysis of histological outcomes. Second, we examined the effect of glucose by separately comparing the saline-asphyxia and glucose-asphyxia groups using two-way ANOVA with group as the independent factor. As above, time was treated as a repeated measure for neurophysiological and biochemical parameters while the five brain regions were used during analysis of histological outcomes. A baseline covariate was used where appropriate.

Similarly, analysis of the adaptation to asphyxia and post-asphyxial seizures were performed by separately comparing the dexamethasone-asphyxia and glucose-asphyxia groups to the saline-asphyxia group by ANOVA with group as the independent factor. For analysis of adaptation to asphyxia, two-way ANOVA was utilized with time treated as a repeated measure. The average of the 60 min immediately before asphyxia was used as a covariate for analysis of cortical impedance.

If a significant effect of the respective independent factors was found, the effect of group was further investigated using the Fisher's protected least significant difference (LSD) post-hoc test. During comparison of the saline-asphyxia and glucose-asphyxia groups, a one-way ANOVA was used to further investigate time points or regions of interest where appropriate. Linear regressions were performed to compare the post-maternal treatment glucose levels with histological and neurophysiological outcomes across all three asphyxia groups. Statistical significance was accepted when $p < 0.05$. Data are presented as mean ± SEM.

## Results

### *The effects of dexamethasone and glucose before asphyxia*

Before asphyxia, dexamethasone ($p < 0.001$) and glucose ($p < 0.001$) treatment were associated with a variable increase in fetal arterial glucose levels (dexamethasone: 1.1–2.7, glucose: 1.5–3.7, saline: 0.8–1.4 mmol/L, Table 1). Dexamethasone was associated with increased MAP compared to the saline-asphyxia group ($42.7 \pm 0.5$ vs. $35.5 \pm 1.0$ mmHg, $p < 0.001$). Dexamethasone ($p < 0.001$) and glucose ($p < 0.01$) treatment were associated with decreased cortical impedance before asphyxia (Figures 1 and 2).

### *Dexamethasone and glucose improve neurophysiological adaptation to asphyxia*

Asphyxia was associated with sustained bradycardia (dexamethasone: $196.9 \pm 4.2$ vs $51.9 \pm 3.9$, glucose: $190.9 \pm 1.9$ vs $53.0 \pm 3.8$ saline: $195.3 \pm 3.1$ vs $60.4 \pm 2.9$ bpm at the end of asphyxia, $p < 0.001$) and severe hypotension (dexamethasone: $42.7 \pm 0.5$ vs $14.4 \pm 1.3$, glucose: $38.0 \pm 1.8$ vs $11.7 \pm 0.5$, saline: $35.5 \pm 1.0$ vs $13.4 \pm 1.5$ mmHg at the end of asphyxia, $p < 0.001$). Two fetuses in each groups had their occlusion ended early (dexamethasone: at 21:19 and 20:42 min, glucose: at 22:32 and 17:30 min, saline: at 23:23 and 20:40 min) due to severe hypotension (MAP < 8 mmHg). Length of asphyxia and final extent of hypotension was not significantly different between the groups.

Cortical impedance increased during asphyxia and peaked $2.4 \pm 0.4$ (saline) and $2.1 \pm 0.3$ (dexamethasone) min after the end of asphyxia (Figure 1). This peak occurred significantly later after the end of asphyxia after glucose treatment ($4.5 \pm 0.8$ min, $p < 0.05$). Compared to the saline-asphyxia group, the rate of rise of cortical impedance was attenuated by dexamethasone from 7 to 15 min ($p < 0.05$) and by glucose treatment from 7 to 21 min ($p < 0.05$) during asphyxia. Glucose treatment attenuated the increase in impedance during final 5 min of asphyxia ($p < 0.05$) while the dexamethasone-asphyxia group was intermediate. Pre-asphyxia glucose levels were inversely correlated with the peak rise in cortical impedance, with higher glucose levels associated with lower maximal cortical impedance ($p < 0.05$, $R^2 = 0.30$, $n = 21$, Supplementary Figure 1). After the post-occlusion peak, impedance progressively resolved after the end of asphyxia in all occlusion groups.

EEG power and SEF were rapidly suppressed during asphyxia (Figure 1) but dexamethasone ($p < 0.05$) and glucose ($p < 0.05$) treatment were associated with higher EEG power compared to saline treatment. SEF was significantly lower in the glucose-asphyxia group compared to the saline-asphyxia group ($p < 0.05$). The raw EEG rapidly became isoelectric in the saline-asphyxia group, while low frequency rolling waveforms were observed with dexamethasone and glucose treatment.

### *Dexamethasone and glucose caused EEG hyperactivity 0–6 h after asphyxia*

Dexamethasone and glucose treatments were associated with increased EEG power (dexamethasone: $p < 0.01$, glucose: $p < 0.05$) and reduced SEF (dexamethasone: $p < 0.001$, glucose: $p < 0.005$, Figure 2) compared to saline treatment during the first 6 h after asphyxia. Additionally, EEG power was higher in the dexamethasone-asphyxia group compared to the glucose-asphyxia group between 4- and 6-h post-asphyxia ($p < 0.05$). Both asphyxia ($p < 0.05$) and dexamethasone ($p < 0.001$) were independently associated with a fall

**Table 1.** Fetal pH, blood gases, and metabolites during the baseline period, asphyxia, and early recovery.

| | Baseline | | Asphyxia | | Early recovery | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Group | Pre-maternal treatment | Post-maternal treatment | 5 min | 17 min | +10 min | +1 h | +2 h | +4 h | +6 h |
| pH | | | | | | | | | |
| SS | 7.38 ± 0.01 | 7.38 ± 0.00 | 7.38 ± 0.01 | 7.38 ± 0.01 | 7.38 ± 0.01 | 7.38 ± 0.01 | 7.38 ± 0.01 | 7.38 ± 0.01 | 7.38 ± 0.01 |
| DS | 7.37 ± 0.01 | 7.35 ± 0.01Φ | 7.34 ± 0.01 | 7.34 ± 0.01 | 7.33 ± 0.01Φ | 7.34 ± 0.01Φ | 7.35 ± 0.01Φ | 7.34 ± 0.01Φ | 7.34 ± 0.01Φ |
| SA | 7.38 ± 0.01 | 7.38 ± 0.01 | 7.02 ± 0.02Ψ | 6.82 ± 0.02Ψ | 7.15 ± 0.01Ψ | 7.30 ± 0.01Ψ | 7.34 ± 0.02Ψ | 7.41 ± 0.01Ψ | 7.42 ± 0.01Ψ |
| DA | 7.37 ± 0.01 | 7.35 ± 0.00Φ | 7.03 ± 0.01Ψ | 6.79 ± 001Ψ | 7.10 ± 0.01ΨΦ | 7.27 ± 0.01ΨΦ | 7.29 ± 0.01ΨΦ | 7.36 ± 0.02ΨΦ | 7.39 ± 0.02ΨΦ |
| GA | 7.36 ± 0.01 | 7.36 ± 0.01 | 7.00 ± 0.01 | 6.80 ± 0.01 | 7.11 ± 0.01# | 7.27 ± 0.01# | 7.31 ± 0.02# | 7.37 ± 0.02# | 7.39 ± 0.01# |
| $P_aCO_2$ (mmHg) | | | | | | | | | |
| SS | 47.0 ± 0.4 | 48.1 ± 1.7 | 43.8 ± 0.9 | 46.0 ± 0.8 | 45.1 ± 0.8 | 45.3 ± 1.2 | 48.1 ± 0.6 | 45.5 ± 0.8 | 47.2 ± 1.3 |
| DS | 49.9 ± 1.0 | 49.7 ± 1.5 | 42.8 ± 1.8 | 43.3 ± 1.8 | 43.1 ± 2.0 | 43.7 ± 1.4 | 45.3 ± 1.6 | 44.4 ± 1.2 | 47.7 ± 1.4 |
| SA | 49.6 ± 1.1 | 48.6 ± 1.1 | 107.7 ± 3.7Ψ | 141.3 ± 4.4Ψ | 54.7 ± 1.6 | 43.5 ± 0.9 | 45.4 ± 1.5 | 43.8 ± 0.7 | 47.7 ± 0.6 |
| DA | 52.0 ± 3.1 | 50.7 ± 2.3 | 91.9 ± 4.8Ψ | 144.5 ± 2.4Ψ | 53.3 ± 3.5 | 45.2 ± 2.9 | 44.2 ± 2.4 | 44.7 ± 2.6 | 45.5 ± 2.6 |
| GA | 51.1 ± 1.0 | 49.3 ± 0.6 | 103.4 ± 2.1 | 141.2 ± 4.5 | 56.3 ± 2.1 | 46.6 ± 0.9 | 45.5 ± 1.2 | 44.8 ± 1.5 | 45.3 ± 0.8 |
| $P_aO_2$ (mmHg) | | | | | | | | | |
| SS | 23.6 ± 1.3 | 25.1 ± 0.5 | 23.6 ± 1.0 | 22.9 ± 1.1 | 23.4 ± 1.2 | 23.2 ± 1.3 | 22.7 ± 1.2 | 21.8 ± 1.4 | 23.6 ± 1.0 |
| DS | 23.9 ± 0.7 | 23.5 ± 1.4 | 24.3 ± 1.3 | 24.1 ± 1.2 | 23.9 ± 1.2 | 23.5 ± 1.3 | 24.3 ± 1.2 | 22.6 ± 1.6 | 23.2 ± 1.5 |
| SA | 24.5 ± 1.1 | 22.6 ± 0.9 | 5.9 ± 0.8Ψ | 7.2 ± 1.0Ψ | 33.1 ± 1.5Ψ | 30.5 ± 1.5Ψ | 26.3 ± 1.7Ψ | 26.5 ± 1.5Ψ | 25.7 ± 1.8Ψ |
| DA | 25.7 ± 1.9 | 24.4 ± 1.1 | 8.3 ± 0.9Ψ | 9.7 ± 0.7Ψ | 35.7 ± 2.5Ψ | 29.9 ± 1.3Ψ | 28.8 ± 1.2Ψ | 26.3 ± 1.4Ψ | 26.6 ± 1.6Ψ |
| GA | 22.1 ± 1.4 | 21.4 ± 0.9 | 8.8 ± 0.6# | 8.9 ± 0.5# | 30.0 ± 1.6 | 25.5 ± 0.9 | 24.3 ± 0.9 | 24.0 ± 1.4 | 24.3 ± 1.4 |
| Lactate (mmol/L) | | | | | | | | | |
| SS | 0.9 ± 0.1 | 0.7 ± 0.0 | 0.8 ± 0.0 | 0.9 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.1 | 1.0 ± 0.1 | 0.9 ± 0.1 | 1.1 ± 0.1 |
| DS | 0.7 ± 0.1 | 1.4 ± 0.1Φ | 1.3 ± 0.2 | 1.4 ± 0.1 | 1.4 ± 0.2Φ | 1.5 ± 0.1Φ | 1.5 ± 0.1Φ | 1.6 ± 0.2Φ | 1.5 ± 0.2Φ |
| SA | 1.1 ± 0.1 | 0.8 ± 0.0 | 4.4 ± 0.2Ψ | 6.9 ± 0.4Ψ | 6.4 ± 0.3Ψ | 4.6 ± 0.2Ψ | 3.8 ± 0.5Ψ | 2.4 ± 0.4Ψ | 2.3 ± 0.3Ψ |
| DA | 0.8 ± 0.1 | 1.4 ± 0.1Φ | 4.0 ± 0.4Ψ | 6.9 ± 0.9Ψ | 7.0 ± 0.3ΨΦ | 5.7 ± 0.4ΨΦ | 5.4 ± 0.4ΨΦ | 5.5 ± 0.5ΨΦ | 5.0 ± 0.6ΨΦ |
| GA | 1.2 ± 0.2 | 1.2 ± 0.2# | 4.7 ± 0.3 | 7.1 ± 0.4 | 6.6 ± 0.3 | 5.1 ± 0.2 | 4.3 ± 0.4 | 3.1 ± 0.5 | 2.6 ± 0.4 |
| Glucose (mmol/L) | | | | | | | | | |
| SS | 1.1 ± 0.1 | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.2 ± 0.1 | 1.1 ± 0.1 | 1.2 ± 0.1 |
| DS | 0.9 ± 0.1 | 1.7 ± 0.1Φ | 1.5 ± 0.1Φ | 1.6 ± 0.1Φ | 1.7 ± 0.1Φ | 1.8 ± 0.1Φ | 1.9 ± 0.1Φ | 1.7 ± 0.2Φ | 1.6 ± 0.1Φ |
| SA | 1.0 ± 0.1 | 1.0 ± 0.0 | 0.3 ± 0.0Ψ | 0.8 ± 0.2Ψ | 1.5 ± 0.2Ψ | 1.3 ± 0.1Ψ | 1.3 ± 0.1Ψ | 1.3 ± 0.1Ψ | 1.5 ± 0.1Ψ |
| DA | 1.0 ± 0.1 | 2.0 ± 0.2Φ | 1.1 ± 0.2ΨΦ | 1.4 ± 0.2ΨΦ | 2.7 ± 0.3ΨΦ | 3.0 ± 0.3ΨΦ | 2.7 ± 0.3ΨΦ | 2.6 ± 0.3ΨΦ | 2.6 ± 0.2ΨΦ |
| GA | 1.0 ± 0.0 | 2.5 ± 0.3# | 2.0 ± 0.4# | 1.9 ± 0.4# | 2.3 ± 0.4 | 1.9 ± 0.3 | 1.6 ± 0.2 | 1.4 ± 0.2 | 1.5 ± 0.2 |

Data are means ± SEM. SS: saline-sham; DS: dexamethasone-sham; SA: saline-asphyxia; DA: dexamethasone-asphyxia; GA: glucose-asphyxia; $P_aCO_2$: arterial pressure of carbon dioxide; $P_aO_2$: arterial pressure of oxygen. Ψ$p < 0.05$, effect of asphyxia; Φ$p < 0.05$, effect of dexamethasone treatment; #$p < 0.05$, glucose-asphyxia vs. saline-asphyxia.

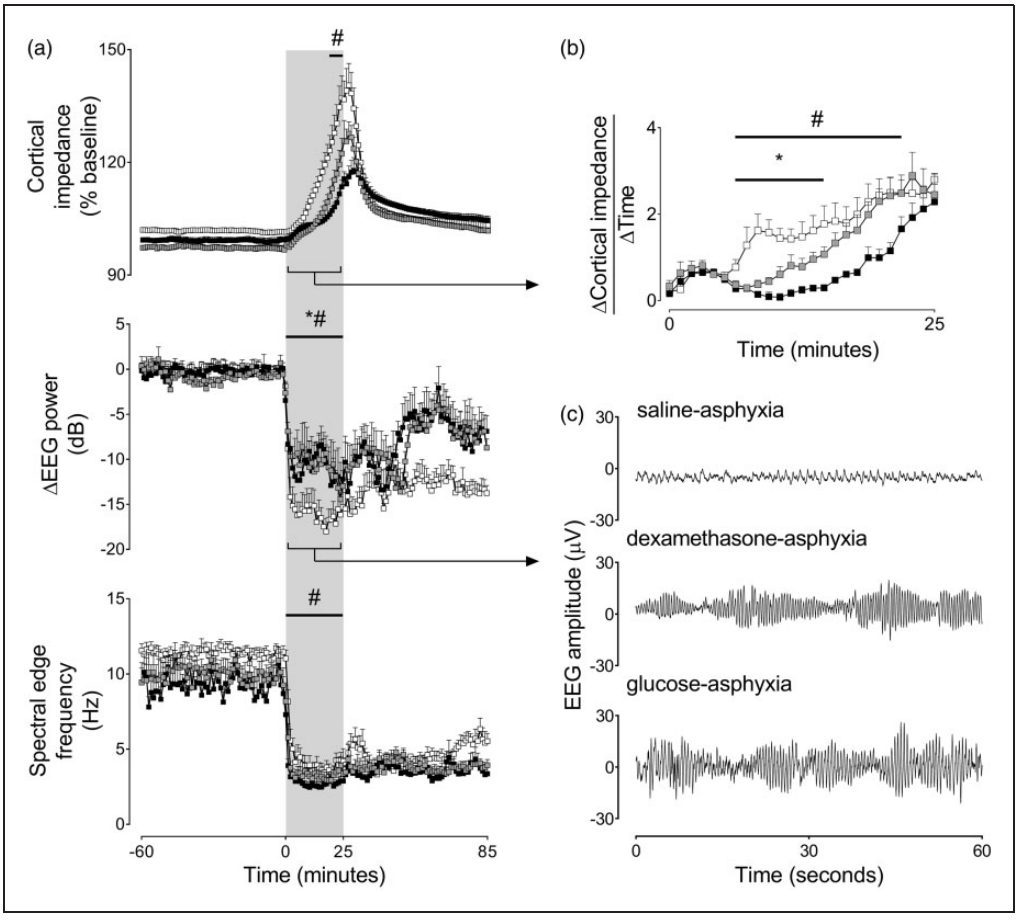


**Figure 1.** (a) Neurophysiological adaptation to asphyxia (1 min mean ± SEM). (b) Rate of change of cortical impedance during asphyxia (1 min mean ± SEM). (c) Raw EEG patterns at approximately 15 min during asphyxia. Note heavy suppression in the saline-asphyxia group and low frequency rolling activity with dexamethasone and glucose treatment. *$p < 0.05$, dexamethasone-asphyxia vs. saline-asphyxia; #$p < 0.05$, glucose-asphyxia vs. saline-asphyxia.

in cortical impedance during the first 6 h after release of umbilical cord occlusion.

### *Dexamethasone and glucose worsened neurophysiological recovery*

Arterial blood gas analysis from day 1 to day 7 after asphyxia is shown in Supplementary Table 1. Saline treatment was associated with stable, near baseline cortical impedance values after asphyxia. In contrast, dexamethasone and glucose treatment were associated with a marked secondary rise in cortical impedance that peaked between 24 and 27 h after asphyxia. In linear regression, higher glucose levels before asphyxia were significantly correlated with a greater maximal rise in cortical impedance during recovery ($p < 0.005$, $R^2 = 0.40$, $n = 21$, Supplementary Figure 2). During the final 72 h of recovery, both asphyxia ($p < 0.001$, Figure 2) and dexamethasone ($p < 0.05$) were associated with a decrease in cortical impedance, and a similar effect was observed in the glucose-asphyxia group ($p < 0.05$). Post-hoc analysis showed that both the dexamethasone-asphyxia ($p < 0.01$) and glucose-asphyxia ($p < 0.05$) groups had reduced cortical impedance compared to the saline-asphyxia group during the final 72 h of recovery.

Recovery of SEF was impaired by dexamethasone treatment with a significant interaction between dexamethasone and asphyxia on SEF from 48 h after asphyxia onwards ($p < 0.01$, Figure 2). Post-hoc analysis showed that in the final 24 h of the experiment, SEF in the saline-asphyxia group had returned to saline-sham and dexamethasone-sham levels, while SEF in the dexamethasone-asphyxia group remained lower than the saline-asphyxia group ($p < 0.001$). A similar effect was observed with glucose treatment, with the glucose-asphyxia group having a lower SEF compared to the saline-asphyxia group from 48 h onwards ($p < 0.001$).

### Dexamethasone and glucose potentiated post-asphyxial seizures

All fetuses in the dexamethasone-asphyxia and glucose-asphyxia groups developed post-asphyxial seizures. One fetus in the saline-asphyxia group did not; occlusion was ended early in this fetus. Dexamethasone and glucose treatment were associated with increased numbers of post-asphyxial seizures (dexamethasone, $389.9 \pm 76.2$; glucose, $300.4 \pm 77.5$; saline, $69.6 \pm 21.6$; $p < 0.005$, Table 2). Seizures after saline treatment predominantly exhibited stereotypical evolving patterns of high amplitude with slow to mixed frequency,[21,33] whereas seizures after dexamethasone and glucose treatment included high-frequency activity (Figure 3). In linear regression, greater glucose levels were significantly correlated with increased numbers of seizures ($p < 0.005$, $R^2 = 0.49$, $n = 22$, Supplementary Figure 2) as well as a lower seizure amplitude ($p < 0.05$, $R^2 = 0.19$, $n = 22$) and duration ($p < 0.01$, $R^2 = 0.32$, $n = 22$).

At the end of the experiment, 4/7 of the dexamethasone-asphyxia and 5/7 of the glucose-asphyxia groups developed burst suppression patterns characterized by a near isoelectric background interrupted by high amplitude bursts (Figure 3). This pattern was never observed in any other group.

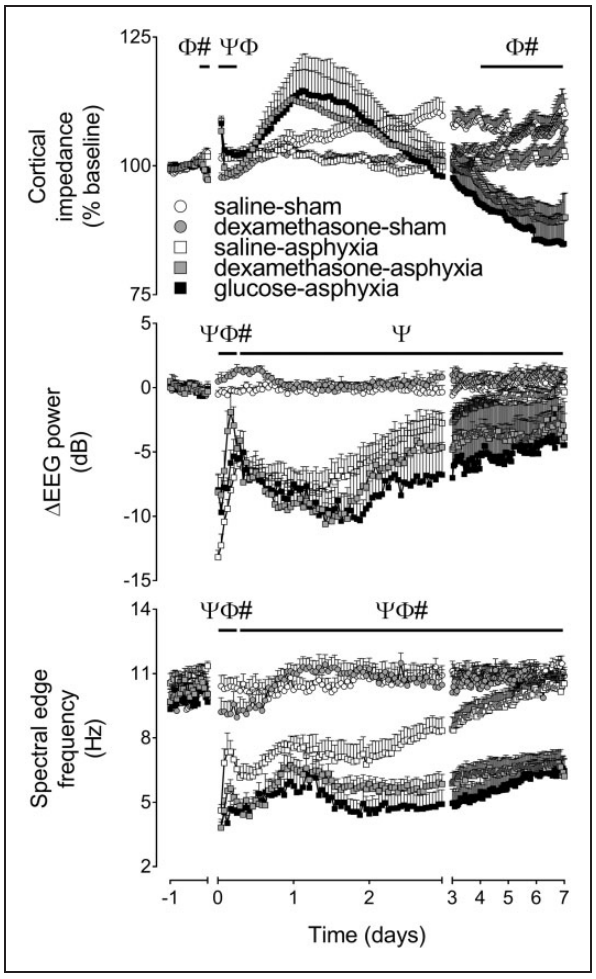


**Figure 2.** Neurophysiological parameters before asphyxia and during recovery. The period of asphyxia is not shown. $\Psi p < 0.05$, effect of asphyxia; $\Phi p < 0.05$, effect of dexamethasone treatment; $\#p < 0.05$, glucose-asphyxia vs. saline-asphyxia.

### Dexamethasone and glucose increased neural injury at 7 days recovery

Both asphyxia and dexamethasone treatment were independently associated with decreased brain weight (asphyxia, $p < 0.001$; dexamethasone, $p < 0.01$, Supplementary Table 2). All fetuses in the saline-sham, dexamethasone-sham, and saline-asphyxia groups displayed intact gross cerebral morphology and no cystic injury. Just one fetus in the saline-asphyxia group showed small cystic lesions in the area surrounding the hippocampus (91.8% of hippocampal area remained intact). The dexamethasone-asphyxia and glucose-asphyxia group consistently developed a spectrum of severe cystic injury in multiple brain regions.

Five regions that showed consistent cystic lesions were then assessed individually: white matter, sagittal gyrus, hippocampus, striatum, and thalamus (Figure 4). There was a significant interaction between dexamethasone and asphyxia on intact area across the five regions ($p < 0.01$, Figure 4). Post-hoc analysis showed that there were no differences between the saline-sham and dexamethasone-sham groups.

**Table 2.** Characteristics of post-asphyxial seizures.

| Group | Seizure onset (h) | Number of seizures | Total seizure period (h) | Max amplitude (μV) | Duration (s) |
|---|---|---|---|---|---|
| SA | $11.0 \pm 4.6$ | $69.6 \pm 21.6$ | $32.7 \pm 6.2$ | $137.7 \pm 16.8$ | $85.5 \pm 11.2$ |
| DA | $8.1 \pm 1.3$ | $389.9 \pm 76.2$* | $74.4 \pm 23.2$ | $96.3 \pm 11.8$ | $62.8 \pm 11.7$ |
| GA | $5.8 \pm 1.7$ | $300.4 \pm 77.5$# | $82.0 \pm 20.9$# | $63.7 \pm 7.0$# | $46.7 \pm 8.5$# |

Data are means ± SEM. SA: saline-asphyxia; DA: dexamethasone-asphyxia; GA: glucose-asphyxia. $*p < 0.05$, dexamethasone-asphyxia vs. saline-asphyxia; $\#p < 0.05$, glucose-asphyxia vs. saline-asphyxia.

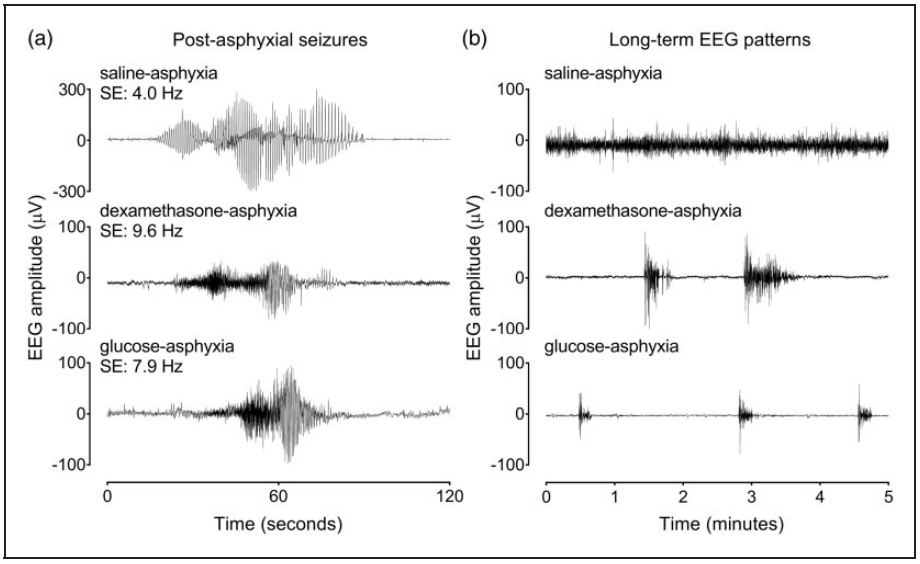


**Figure 3.** (a) Post-asphyxial seizures with corresponding spectral edge frequency (SEF). Note the different scales used. (b) EEG patterns at 7 days recovery.

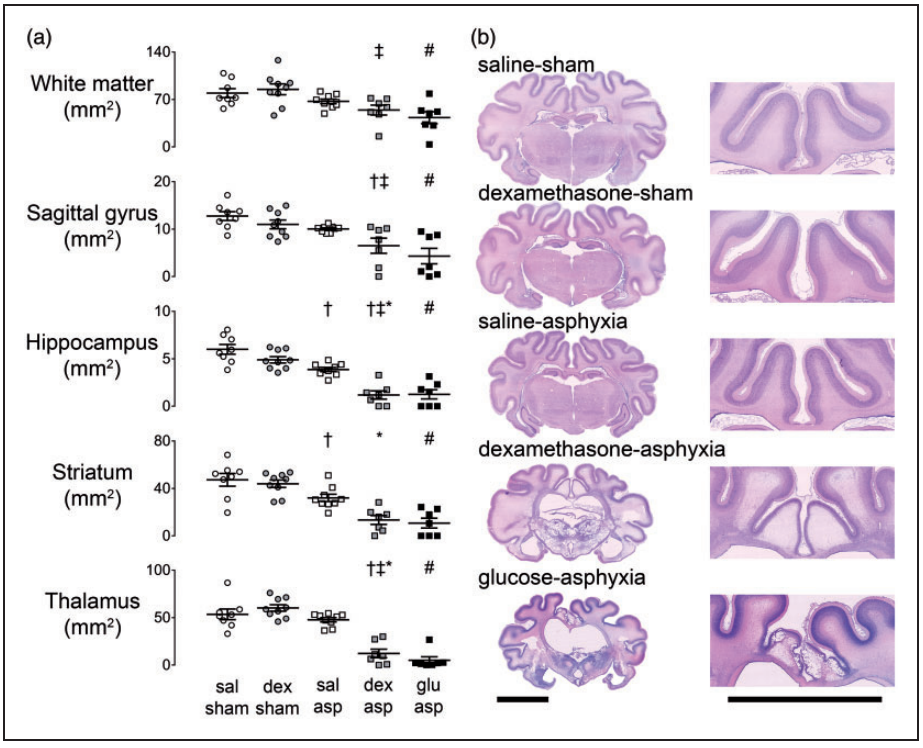


**Figure 4.** (a) Area of intact tissue in five brain regions 7 days after asphyxia. Bars indicate mean ± SEM. †$p < 0.05$, vs. saline-sham; ‡$p < 0.05$, vs. dexamethasone-sham; *$p < 0.05$, saline-asphyxia vs. dexamethasone-asphyxia; #$p < 0.05$, saline-asphyxia vs. glucose-asphyxia. (b) Left panels: coronal histological sections. Note severe injury of the thalamus, white matter and absence of the hippocampus in the dexamethasone-asphyxia and glucose-asphyxia groups. Scale bar = 10 mm. Right panels: enlarged sagittal gyri. Note severe injury of the cortex and intragyral white matter. Scale bar = 10 mm.

The saline-asphyxia group had lower intact area than the saline-sham group in the hippocampus ($p < 0.005$) and the striatum ($p < 0.05$). The dexamethasone-asphyxia group had lower intact area than the dexamethasone-sham group in the white matter ($p < 0.05$) and lower intact area than both saline-sham and dexamethasone-sham groups in the sagittal gyrus ($p < 0.05$), the hippocampus ($p < 0.001$), striatum

($p < 0.001$), and thalamus ($p < 0.001$). The dexamethasone-asphyxia group had lower intact area than the saline-asphyxia group in the hippocampus ($p < 0.001$), striatum ($p < 0.05$), and thalamus ($p < 0.001$). The glucose-asphyxia group had lower intact area than the saline-asphyxia group across all regions (white matter, $p < 0.05$; sagittal gyrus, $p < 0.005$; hippocampus, $p < 0.001$; striatum, $p < 0.005$; thalamus, $p < 0.001$). Linear regression showed that glucose levels before occlusion were significantly correlated with intact area across all regions ($p < 0.05$, $n = 23$, Figure 5).

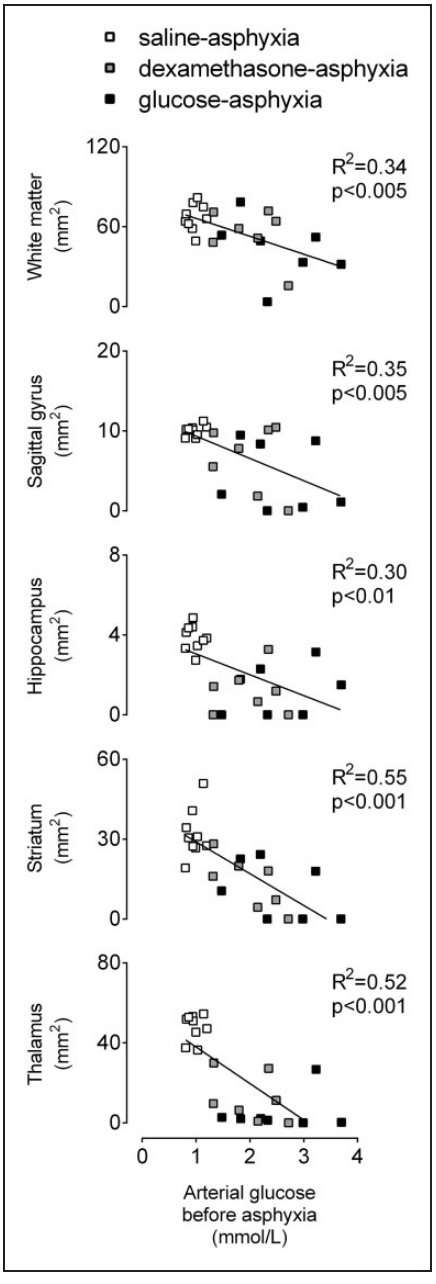


**Figure 5.** Association of increased arterial glucose levels before asphyxia with reduced intact areas.

## Discussion

This study demonstrates that maternal dexamethasone treatment before asphyxia promoted transformation of brain injury from diffuse white and gray matter injury to severe cystic injury. A strikingly similar pattern of injury was observed with glucose infusion before asphyxia, strongly supporting the concept that dexamethasone treatment increased injury at least in part through the induction of fetal hyperglycemia. Collectively this, and previous evidence from term equivalent fetal and neonatal large animal models,[17–20] challenge the findings from postnatal rat studies that found neuroprotection with both dexamethasone[6,7] and hyperglycemia during HI.[11]

In the present study, increased arterial glucose levels improved neurophysiological function during asphyxia. This is consistent with the so-called ''glucose paradox'' in adult species, whereby hyperglycemia is associated with improved metabolic function during HI but subsequent development of greater injury.[34] Thus, our findings contradict the previous proposal that the glucose paradox does not apply to the immature brain.[11] Anoxic depolarization during asphyxia leads to a shift of cations and water from the extracellular space into brain cells. The concomitant increase in resistance to an alternating current (impedance) through the extracellular space provides an index of this cellular edema.[27,35] Dexamethasone and glucose treatment attenuated the rate of rise in cortical impedance and increased EEG activity during asphyxia, consistent with previous findings at term gestation.[36] The maximal increase in impedance was attenuated by glucose treatment. These findings infer that increased glucose supported anaerobic metabolism[36] and so attenuated cellular swelling, consistent with evidence that hyperglycemia reduced ATP depletion during HI in piglets.[37]

Despite this apparent improvement, dexamethasone and glucose treatment were associated with a marked secondary deterioration during recovery from asphyxia, as shown by exacerbated seizures and a delayed increase in cortical impedance that did not occur in vehicle controls. Reversible insults can trigger a biphasic pattern of initial recovery of oxidative metabolism in a ''latent'' phase over approximately 6 h, followed by secondary deterioration with terminal mitochondrial failure from approximately 6 to 48 h after asphyxia,[25,38] and ultimately cell death. During the critical latent phase, cerebral metabolism is consistently suppressed by endogenous neuroinhibitors.[22,39] In contrast, in the present study, dexamethasone and glucose treatment were associated with increased EEG activity in the latent phase, suggesting that hyperglycemia impaired endogenous protective cerebral suppression in the latent phase. This finding is consistent with our findings

that dexamethasone-induced EEG hyperactivity in normoxic preterm fetal sheep,[26] and that dexamethasone treatment after asphyxia was associated with EEG hyperactivity and evidence of increased mitochondrial metabolism on near-infrared spectroscopy.[22] Alternatively, there are data that hyperglycemia can increase expression of cerebral GLUT transporters,[40] and so could contribute to increased metabolism.

At the end of the experiment, the saline-asphyxia group showed recovery of discontinuous EEG patterns. In contrast, SEF remained suppressed in the dexamethasone-asphyxia and glucose-asphyxia groups, in association with the appearance of burst suppression EEG patterns, which are associated with a high risk of mortality and morbidity in human neonates.[41]

Consistent with this evidence of greater secondary deterioration and impaired neurophysiological recovery after asphyxia, histological injury at 7 days after asphyxia was increased by dexamethasone and glucose treatment, with a dramatic shift from diffuse to severe cystic brain injury. Strikingly, infarction was seen even in the sagittal cortex, which we have never observed in fetal sheep at this age. Diffuse, non-cystic white matter injury, with moderate subcortical neuronal loss but sparing of the cortex from acute injury has been found consistently after asphyxia in fetal sheep at this gestation,[21,39,42] consistent with the predominant pattern of periventricular leukomalacia (PVL) in preterm infants.[3] Severe, cystic PVL occurs in approximately 1–3% of preterm infants and is suggested to be the most important risk factor for cerebral palsy among preterm infants.[43] The etiology of cystic PVL is only partly understood[3] but, case reports have associated severe cystic injury with severe prenatal[44,45] and neonatal hyperglycemia.[46]

The overall pattern of injury after dexamethasone and hyperglycemia exposure was a ''reverse watershed'' pattern, with cystic injury particularly involving the sagittal gyrus and thalamus, with relative sparing of the parasagittal gyrus. Asphyxia is commonly associated with a watershed pattern injury, in which the sagittal gyrus has greater residual perfusion and less damage than the parasagittal cortex.[47] Similarly, in near-term fetal sheep during profound asphyxia, thalamic blood flow is preserved compared to the cortex.[48] We speculate that greater perfusion may have facilitated greater glucose delivery and thus more sustained brain activity during asphyxia.

Finally, it is notable that greater glucose levels were associated with increased numbers of seizures. Seizures in the dexamethasone-asphyxia and glucose-asphyxia groups included higher frequency components, and were often of lower amplitude and duration than after saline-asphyxia. This combination is consistent with recruitment of fewer neurons and propagation through shorter neuronal circuits, inferring that they were of cortical rather than subcortical origin and may have contributed to cortical injury.

It is important to appreciate that the present study targeted acute isolated asphyxial injury at a moderately preterm stage of brain development. In the future, it will be interesting to assess the effects of glucocorticoids and hyperglycemia after the complex inflammatory insults that are common in preterm infants.[49]

## Significance and perspectives

The present study highlights potential adverse neural effects of glucocorticoid treatment before perinatal asphyxia. This should not be taken to suggest that we should avoid antenatal glucocorticoids given their considerable overall benefits,[4] but rather that there is an urgent need to better understand the complex interactions that can occur during the perinatal period. There is therefore value in further research to identify obstetric subgroups where the risks of antenatal glucocorticoid treatment might outweigh the potential benefits. Antenatal glucocorticoids were widely introduced without a full appreciation of their non-pulmonary effects. A more in-depth understanding of their side effects will allow us to develop strategies to minimize these risks.

Despite the findings of severe cystic injury in the present study, the rates of cystic injury among preterm infants have fallen over time,[3] despite near universal use of antenatal glucocorticoids. Conversely, it is interesting to note that antenatal glucocorticoids markedly improve short-term outcomes but unexpectedly this has not translated into an overall improvement in neurodevelopmental outcomes.[4] The key finding of the present study is that glucocorticoids sensitized to greater injury. Mild asphyxia is much more common than moderate asphyxia.[50] It is thus plausible that glucocorticoids or hyperglycemia could transform a mild period of asphyxia associated with no significant injury into one that led to diffuse white matter injury. This is an important hypothesis to test in future studies.

Finally, these findings strongly support the potential for hyperglycemia itself to independently exacerbate brain injury. This is of particular concern given that maternal obesity and diabetes are associated with hyperglycemia at all gestations.[51] Conversely, there is considerable clinical emphasis on avoiding hypoglycemia in preterm neonates.[52] Our findings suggest that it is also important to consider the potential for unexpected dangers in allowing permissive hyperglycemia when trying to prevent hypoglycemia.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Health Research Council of New Zealand (grant numbers 12/613 and 14/216), the Auckland Medical Research Foundation (grant number 1108004), and New Zealand Lottery Grants Board (grant numbers 209214 and 340855). Christopher Lear was supported by an Auckland Medical Research Foundation Doctoral Scholarship (grant number 1213003).

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Authors' contributions

These experiments were conducted in the Fetal Physiology and Neuroscience Group laboratory, at the University of Auckland. C.A.L., L.B., and A.J.G. conceived the hypotheses, experimental design, and analysis protocols for this study. C.A.L., J.O.D., P.P.D., R.G., J.S.Q., and L.B. were responsible for data collection. C.A.L. performed the histology and microscopy analysis. C.A.L. and G.R.M. performed the physiological analysis. C.A.L. drafted the manuscript. 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.

## Supplementary material

Supplementary material for this paper can be found at the journal website: http://journals.sagepub.com/home/jcb

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