Hypothermic Neuroprotection Is Associated With Recovery of Spectral Edge Frequency After Asphyxia in Preterm Fetal Sheep

Guido Wassink, MSc*; Robert D. Barrett, PhD*; Joanne O. Davidson, PhD; Laura Bennet, PhD; Robert Galinsky, PhD; Michael Dragunow, PhD; Alistair J. Gunn, MBChB, PhD

Background and Purpose—Electroencephalographic recovery is predictive of outcome after perinatal hypoxia–ischemia, but it is unknown whether early changes in electroencephalographic can predict the response to therapeutic hypothermia in the preterm brain.

Methods—0.7 gestation fetal sheep received umbilical cord occlusion or sham occlusion for 25 minutes, followed by sham hypothermia or whole-body cooling started either 30 minutes or 5 hours after occlusion and continued for 72 hours.

Results—Early but not delayed hypothermia reduced neuronal loss and microglial induction in the striatum, with faster recovery of spectral edge frequency, reduced seizure burden, and less suppression of electroencephalographic amplitude (P<0.05).

Conclusions—Recovery of higher electroencephalographic frequencies may be a biomarker of effective hypothermic neuroprotection in the preterm-equivalent brain. (Stroke. 2015;46:585-587. DOI: 10.1161/STROKEAHA.114.008484.)

Key Words: EEG ■ hypothermia

Mild induced hypothermia improves intact recovery in term infants with moderate to severe hypoxic–ischemic encephalopathy but has not been tested in preterm infants. Early electroencephalographic (EEG) monitoring is predictive of long-term outcome after perinatal hypoxia–ischemia at term, but the effect of mild hypothermia on EEG recovery in preterm infants is unknown. In normal humans, mild hypothermia (to ≈33.5°C) is associated with a small reduction in EEG amplitude, with a minor shift in frequencies to theta and beta activity. In contrast, mild hypothermia after traumatic brain injury in rats increased the proportion of isoelectric EEG, and after pediatric cardiac arrest, worsening of EEG background abnormalities during hypothermia was associated with adverse outcomes. Thus, the severity of brain injury may modulate the effects of hypothermia on brain activity.

In the present study, we examined the hypothesis that early induction of hypothermia after asphyxia in preterm (0.7 gestational) fetal sheep would be associated with improved striatal neuroprotection and EEG recovery compared with hypothermia delayed for 5 hours, just before the typical onset of postasphyxial seizures. At this age, brain development is broadly consistent with 28 to 32 weeks gestation in humans.

Methods
Detailed methods are provided in the online-only Data Supplement.

Surgical Procedures
All procedures were approved by the Animal Ethics Committee of The University of Auckland. Fetal sheep at 97 to 99 days gestation (term=147 days) were instrumented with brachial artery catheters and extradural EEG electrodes. Thermistors were placed over the parasagittal dura and in the esophagus. An occluder was fitted around the umbilical cord and a cooling coil was tied over the fetal back.

Experimental Proceedings
At 103 to 104 days gestation, fetuses were randomly assigned to sham occlusion followed by normothermia (sham-normothermia, n=8), or whole-body cooling for 72 hours (sham-hypothermia, n=8), or umbilical cord occlusion for 25 minutes, followed by sham hypothermia (occlusion-normothermia, n=12), or whole-body cooling started from 30 minutes (occlusion-early hypothermia, n=10), or 5 hours (occlusion-delayed hypothermia, n=7) after occlusion and continued for 72 hours. Mild whole-body cooling was induced by circulating cold water through the cooling coil. Seven days after occlusion, the ewes and fetuses were killed.

Mean arterial pressure, blood gases, EEG activity, and temperature were recorded throughout the experiment. EEG power and spectral
edge frequency (SEF) were calculated. Electroencephalographic seizures were identified. Data are mean± SEM.

**Immunohistochemistry**
Brain sections were stained for NeuN (neuronal survival) and IB4 (activated microglia) in the caudate nucleus and putamen, and positive cells were counted stereologically.

**Statistical Analysis**
Data were evaluated by repeated measures ANCOVA (SPSS v22, SPSS Inc, IL) and Sidak post hoc analysis. The within subjects’ correlation was assessed between EEG amplitude (μV) and extradural temperature. Statistical significance was accepted at $P<0.05$.

**Results**

**Blood Composition, Arterial Pressure, and Extradurral Temperature**
Umbilical cord occlusion was associated with profound hypoxia, mixed metabolic and respiratory acidosis, and hypotension, with rapid recovery after release of occlusion. Hypothermia was associated with a small increase in pH and glucose and a lower PaCO2 compared with occlusion-normothermia (Table I in the online-only Data Supplement) and no effect on mean arterial pressure (Table II in the online-only Data Supplement). Fetal extradural temperatures are shown in Figure 1.

**EEG Power and SEF**
Occlusion was associated with suppressed EEG power until 84 hours after asphyxia in the occlusion-normothermia group and 72 hours in both hypothermia groups ($P<0.05$; versus sham-normothermia; Figure 1). Compared with occlusion-normothermia, EEG power was reduced from 1 to 12 hours with early hypothermia, and 6 to 12 hours with delayed hypothermia ($P<0.05$). Delayed hypothermia was associated with suppressed EEG power compared with early hypothermia from 24 to 30 hours ($P<0.05$).

SEF was significantly suppressed with occlusion-normothermia until 72 hours after occlusion compared with sham-normothermia ($P<0.05$; Figure 1). In contrast, early hypothermia showed rapid recovery of SEF to sham-normothermia values within 4 hours, with higher SEF than delayed hypothermia from 30 to 72 and 78 to 162 hours ($P<0.05$). Examples of continuous EEG are shown in Figure I in the online-only Data Supplement.

**Relationship Between Extradural Temperature and EEG Amplitude**
All groups showed a significant relationship between extradural temperature and EEG amplitude (Figure II in the online-only Data Supplement). Delayed hypothermia was associated with a greater effect on EEG amplitude ($2.16±0.79$ μV/°C, $r=0.64$, $P<0.001$) than early hypothermia ($0.53±0.08$ μV/°C, $r=0.50$, $P<0.001$).

**Seizures**
Numbers of seizures, seizure burden (total min of seizure activity), and mean seizure duration during recovery were reduced after early but not delayed hypothermia ($P<0.05$; Table III in the online-only Data Supplement).

**Striatal Neurons and Microglia**
Occlusion was associated with significant loss of neurons in the caudate and putamen ($P<0.05$; versus sham-normothermia; Figure 2 and Figure III in the online-only Data Supplement). Neuronal survival was increased after early but not delayed hypothermia in the caudate nucleus. Both occlusion-hypothermia groups showed intermediate neuronal survival in the putamen between sham-normothermia and occlusion-normothermia.

Occlusion was associated with intense induction of microglia in the caudate and putamen ($P<0.05$; versus sham-normothermia; Figure 2 and Figure III in the online-only Data Supplement). This was markedly reduced after early hypothermia ($P<0.05$), but only partially suppressed after delayed hypothermia.

**Discussion**
This study demonstrates that mild whole-body cooling in preterm fetal sheep, started within 30 minutes after severe
asphyxia, was associated with faster recovery of SEF to sham control values in the first 4 hours, and subsequently markedly reduced seizure burden and less suppression of EEG power and amplitude, than hypothermia delayed until 5 hours after asphyxia. Consistent with this, early but not delayed cooling was associated with improved striatal neuronal survival and suppression of microglia induction after 7 days recovery. These findings raise the possibility that delayed recovery of SEF could help identify infants who benefit less from hypothermia.

Prolonged umbilical cord occlusion was associated with moderate to severe subcortical neural injury but sparing of the cortex after 3 days recovery, and with prolonged suppression of EEG power and amplitude, than hypothermia delayed until 5 hours after asphyxia. Consistent with this, early but not delayed cooling was associated with improved striatal neuronal survival and suppression of microglia induction after 7 days recovery. These findings raise the possibility that delayed recovery of SEF could help identify infants who benefit less from hypothermia.

The finding that early cooling was associated with reduced seizures but less suppression of overall EEG amplitude/power than delayed cooling strongly suggests an indirect effect, mediated by neuronal protection. We speculate that early recovery of SEF and reduced suppression of EEG amplitude during cooling may be biomarkers of clinical response to therapeutic hypothermia.

Sources of Funding

This study was funded by the Health Research Council of New Zealand and Gravida. Mr Guido Wassink was supported by the University of Auckland Health Research Doctoral Scholarship. Dr Barrett was supported by a scholarship from the William Georgetti Charitable Trust.

Disclosures

None.

References


Figure 2. Numbers of IB4-positive microglia cells (A) and NeuN-positive neurons (B) in the caudate and putamen 7 days after sham occlusion in preterm fetal sheep. Data are mean±SEM. *P<0.05, vs sham-normothermia; #P<0.05, occlusion-hypothermia groups vs occlusion-normothermia.
Hypothermic Neuroprotection Is Associated With Recovery of Spectral Edge Frequency After Asphyxia in Preterm Fetal Sheep
Guido Wassink, Robert D. Barrett, Joanne O. Davidson, Laura Bennet, Robert Galinsky, Michael Dragunow and Alistair J. Gunn

Stroke. 2015;46:585-587; originally published online January 13, 2015; doi: 10.1161/STROKEAHA.114.008484

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/46/2/585

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2015/01/30/STROKEAHA.114.008484.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Hypothermic neuroprotection is associated with recovery of spectral edge frequency after asphyxia in preterm fetal sheep

Guido Wassink, PhD¹; Robert D. Barrett, PhD¹; Joanne O. Davidson, PhD¹; Laura Bennet, PhD¹; Robert Galinsky, PhD¹; Michael Dragunow, PhD²; Alistair J. Gunn, MBChB, PhD¹.

1. Department of Physiology, University of Auckland, New Zealand
2. Department of Anatomy with Radiology, University of Auckland, New Zealand
Supplemental Methods

Experimental preparation

All animal procedures were approved by the Animal Ethics Committee of The University of Auckland, New Zealand. We have previously reported changes in temperature, carotid blood flow and serum nitrate values in the sham and early hypothermia groups.\(^1\) Time-mated singleton Romney/Suffolk fetal sheep from the university approved farm, were instrumented between 97 and 99 days gestation (term = 147 days). Food, but not water was withdrawn 18 hours before surgery. Ewes were given 5 mL of Streptocin (procaine penicillin (250,000 IU) and dihydrostreptomycin (250 mg.mL\(^{-1}\)), Stockguard Labs, Hamilton, New Zealand) intramuscularly for prophylaxis 30 min prior to the start of surgery. Anesthesia was induced by intravenous injection of Alfaxan (alphaxalone, 3 mg.kg\(^{-1}\); Jurox, Rutherford, NSW, Australia), and after intubation, general anesthesia maintained using 2-3% isoflurane in oxygen. The depth of anesthesia and maternal respiration were constantly monitored by trained staff during surgery. A 20 gauge catheter was placed in a maternal front leg vein to provide a constant infusion of isotonic saline (250 mL.h\(^{-1}\)).

All surgical procedures were performed using sterile techniques as previously described.\(^2\) Catheters were placed in the left and right brachial artery for preductal blood sampling and arterial blood pressure measurements respectively. An amniotic catheter was secured to the fetal shoulder. Two pairs of electroencephalogram (EEG) electrodes (AS633-5SSF, Cooner wire Co., Chatsworth, CA, USA) were placed on the dura over the parasagittal parietal cortex (5 mm and 10 mm anterior to bregma and 5 mm lateral), with a reference electrode sewn over the occiput. A thermistor (IncuTemp-1, Mallinckrodt Medical Inc., MO, USA) was placed over the parasagittal dura 20 mm anterior to bregma for the measurement of fetal extradural temperature, and a second thermistor was placed deep in the esophagus at the level of the right atrium to measure fetal core body temperature. An inflatable silicone occluder was placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, CA, USA), and a silicone cooling coil (external diameter, 7.9 mm; internal diameter, 4.8 mm; Degania Silicone, Regensburg, Germany) was tied over the fetal back to allow circulation of water.

The uterus was then closed, after gentamicin (gentamicin sulphate, 40 mg/mL, Rousell, Auckland, New Zealand) was injected into the amniotic sac. Any amniotic fluid lost during surgery was replaced with isotonic saline warmed to 37°C. The maternal laparotomy incision was repaired and the skin infiltrated with the local acting analgesic Marcain (10 mL, 0.5% bupivacaine plus epinephrine, AstraZeneca, Auckland, New Zealand). The maternal long saphenous vein was catheterized for post-operative maternal care, and all fetal leads were exteriorized through the maternal flank. After surgery, sheep were housed together in separate metabolic cages with access to water and concentrate pellet feed (Country Harvest Stockfeed, Cambridge, New Zealand) \textit{ad libitum}. The animal housing facility was temperature-controlled (16 ± 1°C, humidity 50 ± 10%), and operated on a 12 hour light/dark cycle at all times. During the post-operative recovery period before experiments, all ewes were given daily i.v. antibiotics, including gentamicin (40 mg/mL for 2 days, Rousell) and benzylpenicillin sodium (600 mg for 4 days, Novartis Ltd., Auckland, New Zealand). Fetal vascular catheters were maintained patent by continuous infusion of heparinized saline (20 U/mL at 0.15 mL.h\(^{-1}\)), and the maternal catheter was maintained by daily flushing.

Experimental recordings

Experiments were conducted at 103-104 days gestation. Fetal mean arterial pressure (MAP; (Novatrans II, MX860, Medex Inc., Hilliard, OH, USA), corrected for maternal movement by subtraction of amniotic fluid pressure, EEG activity and temperature were recorded
continuously from 12 hours before the experiment until 168 hours afterwards. The blood pressure signals were collected at 64 Hz and low pass filtered at 30 Hz. The EEG signal was processed with a first-order high-pass filter at 1.6 Hz and a 6th order Butterworth low-pass filter with a cut-off frequency at 50 Hz, and then digitally stored at a sampling rate of 64 Hz. The EEG power (dB) and spectral edge frequency (SEF) were then calculated on the portion of the power spectrum between 1 Hz and 20 Hz. The EEG power (dB) signal was log transformed (decibels (dB), \( 20 \times \log(\text{intensity}) \)), as this transformation gives a better approximation of normal distribution, and data from left and right EEG electrodes were averaged. The SEF was defined as the frequency below which 90% of the EEG intensity lies. All experimental data were collected by computers with custom software (Labview for Windows, National Instruments Ltd, Austin, TX, USA).

**Experimental protocols**

Fetuses were randomly assigned to either sham-normothermia (n = 8), sham-hypothermia (n = 8), occlusion-normothermia (n = 12), occlusion-early hypothermia (n = 10), or occlusion-delayed hypothermia (n = 7). Fetal asphyxia was induced for 25 min by rapid inflation of the umbilical cord occluder with sterile saline of a defined volume known to completely inflate the occluder. Successful occlusion was confirmed by rapid onset of bradycardia and changes in blood gases. Fetal arterial blood was taken 60 min before occlusion, at 5 and 17 min during occlusion, and 10 min, 1, 2, 4, 6, 24, 48, 72, 96, 120, 144 and 168 hours after occlusion for blood gas, glucose and lactate determination (ABL800 Flex analyzer, Radiometer, Auckland, New Zealand). Blood gases for the hypothermia groups were temperature corrected.

Intrauterine whole body cooling was initiated either 30 min (early hypothermia) or 5 hours (delayed hypothermia) after the end of occlusion and continued for 72 hours. At the end of cooling, fetuses were allowed to rewarm spontaneously. Cooling was induced by circulating cold water (10ºC) through the cooling coil around the fetal body, and was titrated in the first 2 hours to reduce fetal extradural temperature from 39.4 ± 0.1ºC to between 36 and 37ºC. Seven days after occlusion the ewes and fetuses were killed by an overdose of sodium pentobarbitone (9 g, i.v. to the ewe; Pentobarb 300, Chemstock International, Christchurch, New Zealand). The fetal brains were perfusion fixed in situ with saline followed by 500 mL of 10% phosphate buffered formalin. Following removal from the skull, brain tissue was fixed for another 5-6 days before processing and embedding using a standard paraffin preparation. One animal from the sham-hypothermia group was euthanized one day before its scheduled post-mortem date due to acute umbilical cord entanglement. This fetus was otherwise healthy throughout the experiment (as assessed by blood gases) but its histological data was excluded from subsequent analysis.

**Immunohistochemistry**

Slides were cut (6 µm thick) using a microtome (Leica Jung RM2035, Wetzlar, Germany). Slides were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Slides were washed in 0.1mol/L phosphate buffered saline (PBS). Antigen retrieval was performed using the citrate buffer boil method followed by incubation in 1% H2O2 in methanol. For antineuronal nuclei monoclonal antibody (NeuN, a neuronal marker), blocking was performed in 2.5% normal horse serum (NHS) for 1 hour at room temperature, followed by mouse anti-NeuN (1:400, Chemicon International, Temecula, CA, USA) in PBS plus 2.5% NHS overnight at 4ºC. Biotin-conjugated secondary antibody (1:200 in PBS plus 2.5% NHS, overnight at 4ºC), avidin-biotin complex (1:50, 2 hours at room temperature; Vectastain Elite ABC Kit, Vector Laboratories), and 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, Sydney, AU) were used to visualize the positive signal. For negative control sections
the primary antibody was omitted from the incubation solution. Staining was performed in separate batches, with each batch containing two sections from each experimental group.

For activated microglia, rehydrated/antigen retrieved sections were incubated with biotinylated isoelectin B4 (IB4, Sigma-Aldrich Pty. Ltd., Sydney, AU) overnight at 4°C, diluted 1:100 in PBS. Sections were then washed 3 x 5 min in PBS, incubated in avidin-biotin complex (1:50, 2 hours at room temperature; Vector Laboratories), washed in PBS (3 x 5 min), and then visualized using DAB (Sigma-Aldrich Pty, Ltd.). The reaction was terminated by washing in distilled water, and the sections dehydrated and mounted. For negative control sections the primary antibody was omitted from the incubation solution.

For each animal, four serial sections of the forebrain were analyzed taken 23 mm anterior to stereotaxic zero at the level of the mid-striatum (comprising the caudate and putamen). NeuN and IB4 positive cells were counted on stained sections by light microscopy on a Nikon eclipse 80i microscope with motorized stage (Scitech Pty. Ltd., Preston, Victoria, Australia) and Stereo Investigator software (version 8; MicroBrightField Inc., Williston, VT, USA), by an investigator who was masked to the treatment groups by separate coding of the slides. Sampling was performed using stereological principles by first tracing around each region of interest at 2 x magnification, and then randomly translating a grid onto the sections and applying an optical fractionator probe consisting of a counting frame for object inclusion/exclusion at 40x magnification. The grid and counting frame sizes used for the caudate and putamen were 600 x 600 μm and 100 x 100 μm, respectively. Cells touching the bottom and right-hand boundaries were included, whereas those touching the top and left were excluded. For the caudate and putamen, the estimated total number of cells (slides were cut at 6 μm thickness, with 1 slide every 10 sampled giving a total structure thickness of 240 μm), was calculated using Stereo Investigator (MicroBrightField).

**Data analysis and statistics**

All physiological analyses were performed using custom analysis programs (Labview for Windows), by an investigator blinded to the treatment groups through coding of all experimental animals. Overt electrographic seizures were identified visually and defined as the concurrent appearance of sudden, repetitive, evolving stereotyped waveforms in the EEG signal, lasting more than 10 seconds and >20 μV, as previously described by Scher et al.5 Seizure burden was calculated as the sum duration of all seizures after occlusion. Signal artefact precluded seizure analysis in 3 and 2 fetuses in the occlusion-normothermia and early hypothermia groups respectively. The effect of occlusion and hypothermia on fetal physiological parameters was evaluated by analysis of variance, with time as a repeated measure and baseline values as a covariate where appropriate (SPSS v22, SPSS Inc., Chicago, IL, USA). Sidak post-hoc testing was performed on 6 hour averaged time blocks when a significant overall effect of group, or an interaction between group and time was found. Blood composition, seizure, and histological data were assessed by univariate analysis and the Sidak post-hoc test. Statistical significance was accepted at p<0.05. Data are presented as mean ± standard error of the mean (SEM).

We analyzed the Pearson within subjects’ correlation between EEG amplitude (μV) and extradural temperature (°C) over 96 hourly observations from the end of occlusion, using the method of Bland & Altman.6 EEG amplitude was used instead of power because continuous EEG amplitude monitoring is more commonly and immediately available.7
## Supplemental Tables

### Table I. Fetal Biochemical Changes.*

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>5 Min</th>
<th>17 Min</th>
<th>+10 Min</th>
<th>+1 Hr</th>
<th>+2 Hrs</th>
<th>+4 Hrs</th>
<th>+6 Hrs</th>
<th>+24 Hrs</th>
<th>+72 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SN</td>
<td>7.38±0.01</td>
<td>7.38±0.01</td>
<td>7.38±0.01</td>
<td>7.38±0.01</td>
<td>7.38±0.01</td>
<td>7.38±0.01</td>
<td>7.38±0.01</td>
<td>7.37±0.00</td>
<td>7.37±0.00</td>
<td></td>
</tr>
<tr>
<td>SH</td>
<td>7.37±0.01</td>
<td>7.36±0.01</td>
<td>7.37±0.01</td>
<td>7.39±0.01</td>
<td>7.39±0.01</td>
<td>7.41±0.01</td>
<td>7.40±0.01</td>
<td>7.39±0.01</td>
<td>7.40±0.01</td>
<td></td>
</tr>
<tr>
<td>ON</td>
<td>7.36±0.00</td>
<td>7.04±0.01*</td>
<td>6.86±0.01*</td>
<td>7.13±0.03*</td>
<td>7.28±0.02*</td>
<td>7.34±0.02</td>
<td>7.42±0.01</td>
<td>7.40±0.00</td>
<td>7.37±0.01</td>
<td></td>
</tr>
<tr>
<td>EH</td>
<td>7.35±0.01</td>
<td>7.04±0.02*</td>
<td>6.85±0.01*</td>
<td>7.13±0.02*</td>
<td>7.30±0.02*</td>
<td>7.38±0.01</td>
<td>7.43±0.01*</td>
<td>7.45±0.01*#</td>
<td>7.42±0.01*#</td>
<td>7.41±0.01*#</td>
</tr>
<tr>
<td>DH</td>
<td>7.36±0.02</td>
<td>6.97±0.01*#</td>
<td>6.80±0.01*#</td>
<td>7.15±0.02*</td>
<td>7.29±0.01*</td>
<td>7.33±0.02</td>
<td>7.39±0.01</td>
<td>7.42±0.01</td>
<td>7.42±0.01*#</td>
<td>7.42±0.01*#</td>
</tr>
</tbody>
</table>

| PaCO₂ mmHg |          |       |        |         |       |        |        |        |         |         |
| SN         | 46.7±0.3 | 43.9±0.9| 45.8±0.8| 45.0±0.8| 44.5±1.3| 48.2±0.6| 46.1±1.1| 47.4±1.5| 48.5±0.5| 49.6±0.7|
| SH         | 48.8±1.0 | 43.9±1.4| 44.0±1.3| 43.0±1.1| 40.6±1.0| 44.5±1.2| 40.2±0.9| 44.0±1.5| 44.6±0.7| 48.9±2.1|
| ON         | 49.2±1.1 | 91.7±3.4*| 128.2±6.6*| 60.2±8.7| 43.7±1.2| 46.6±1.0| 44.4±0.9| 46.6±1.2| 44.7±1.2| 45.4±1.3|
| EH         | 48.6±1.3 | 93.9±2.7*| 124.7±1.6*| 53.2±3.9| 39.6±1.4| 41.5±1.3*#| 39.0±1.3*#| 39.3±1.8*#| 42.5±1.4*| 47.6±1.2|
| DH         | 46.6±1.4 | 101.8±3.0*| 133.3±3.2*| 49.3±2.7| 41.4±1.5| 44.8±1.9| 43.1±1.1| 41.0±1.0*| 42.1±1.3*| 43.0±0.8*|

<p>| PaO₂ mmHg |          |       |        |         |       |        |        |        |         |         |
| SN         | 23.3±1.2 | 23.4±1.0| 22.7±1.1| 22.9±1.0| 22.6±1.3| 22.4±1.1| 21.5±1.3| 23.2±1.1| 22.8±1.0| 23.7±1.4|
| SH         | 25.2±1.2 | 25.7±1.2| 24.9±1.1| 25.1±1.2| 22.8±1.2| 22.9±1.0| 23.4±1.0| 23.4±1.0| 22.4±1.2| 20.1±1.1|
| ON         | 22.3±0.6 | 5.6±0.5*| 7.9±0.7*| 31.2±1.7*| 28.0±1.0*| 24.3±0.8| 23.3±1.1| 22.5±1.0| 25.2±1.0| 26.0±0.9|</p>
<table>
<thead>
<tr>
<th></th>
<th>EH</th>
<th>DH</th>
<th>SN</th>
<th>SH</th>
<th>ON</th>
<th>EH</th>
<th>DH</th>
<th>SN</th>
<th>SH</th>
<th>ON</th>
<th>EH</th>
<th>DH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate mmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SN</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.8±0.0</td>
<td>0.8±0.0</td>
<td>0.8±0.0</td>
<td>0.8±0.0</td>
<td>0.8±0.0</td>
<td>0.8±0.0</td>
<td>0.8±0.0</td>
<td>0.8±0.0</td>
</tr>
<tr>
<td>SH</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>ON</td>
<td>0.8±0.0</td>
<td>3.6±0.2</td>
<td>5.9±0.2</td>
<td>5.4±0.3</td>
<td>4.4±0.4</td>
<td>3.6±0.5</td>
<td>1.8±0.3</td>
<td>1.5±0.2</td>
<td>1.2±0.1</td>
<td>0.9±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EH</td>
<td>0.8±0.1</td>
<td>3.6±0.3</td>
<td>5.0±0.6</td>
<td>5.2±0.3</td>
<td>4.2±0.2</td>
<td>3.3±0.3</td>
<td>1.9±0.3</td>
<td>1.7±0.3</td>
<td>1.2±0.2</td>
<td>0.9±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH</td>
<td>0.8±0.1</td>
<td>4.2±0.2</td>
<td>6.2±0.3</td>
<td>5.3±0.3</td>
<td>3.3±0.4</td>
<td>3.5±0.4</td>
<td>1.8±0.3</td>
<td>1.7±0.4</td>
<td>1.5±0.2</td>
<td>1.0±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose mmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SN</td>
<td>1.1±0.1</td>
<td>1.1±0.1</td>
<td>1.1±0.1</td>
<td>1.0±0.1</td>
<td>1.1±0.1</td>
<td>1.2±0.1</td>
<td>1.1±0.1</td>
<td>1.2±0.1</td>
<td>1.1±0.1</td>
<td>1.0±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
<td>0.9±0.1</td>
<td>1.0±0.1</td>
<td>1.1±0.1</td>
<td>1.4±0.1</td>
<td>1.2±0.1</td>
<td>1.3±0.1</td>
<td>1.1±0.1</td>
<td>1.2±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ON</td>
<td>1.0±0.0</td>
<td>0.3±0.0</td>
<td>0.7±0.1</td>
<td>1.5±0.1</td>
<td>1.4±0.1</td>
<td>1.3±0.1</td>
<td>1.2±0.1</td>
<td>1.3±0.1</td>
<td>1.2±0.1</td>
<td>1.1±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EH</td>
<td>1.1±0.1</td>
<td>0.5±0.0</td>
<td>0.8±0.1</td>
<td>1.7±0.1</td>
<td>1.7±0.1</td>
<td>1.7±0.1</td>
<td>1.6±0.1</td>
<td>1.8±0.0</td>
<td>1.7±0.2</td>
<td>1.6±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH</td>
<td>1.1±0.1</td>
<td>0.4±0.0</td>
<td>0.8±0.1</td>
<td>1.6±0.1</td>
<td>1.2±0.1</td>
<td>1.4±0.1</td>
<td>1.3±0.1</td>
<td>1.5±0.2</td>
<td>1.8±0.1</td>
<td>1.6±0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Fetal arterial pH, blood gases, lactate and glucose values for the sham-normothermia (SN), sham-hypothermia (SH), occlusion-normothermia (ON), occlusion-early hypothermia (EH) and occlusion-delayed hypothermia (DH) groups, 60 min before occlusion (baseline), 5 and 17 min during occlusion, and post (10 min, 1, 2, 4, 6, 24, and 72 hours) 25 min umbilical cord occlusion. Data are mean ± SEM; 48 hours and post 96 hours were not significant and have been omitted for space reasons. *p<0.05; vs. sham-normothermia; #p<0.05; vs. occlusion-normothermia.
Table II. Mean Arterial Blood Pressure Changes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>+3 Hrs</th>
<th>+6 Hrs</th>
<th>+9 Hrs</th>
<th>+12 Hrs</th>
<th>+18 Hrs</th>
<th>+24 Hrs</th>
<th>+48 Hrs</th>
<th>+72 Hrs</th>
<th>+168 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN</td>
<td>37.8 ± 1.0</td>
<td>37.8 ± 0.9</td>
<td>37.1 ± 1.4</td>
<td>37.0 ± 1.5</td>
<td>36.8 ± 1.5</td>
<td>36.9 ± 1.4</td>
<td>37.0 ± 1.2</td>
<td>37.3 ± 1.2</td>
<td>37.2 ± 1.2</td>
<td>39.3 ± 1.8</td>
</tr>
<tr>
<td>SH</td>
<td>35.2 ± 0.9</td>
<td>36.5 ± 1.1</td>
<td>36.5 ± 1.1</td>
<td>36.3 ± 1.1</td>
<td>35.4 ± 1.2</td>
<td>35.0 ± 1.2</td>
<td>35.4 ± 1.5</td>
<td>35.5 ± 1.3</td>
<td>34.5 ± 1.4</td>
<td>37.6 ± 1.9</td>
</tr>
<tr>
<td>ON</td>
<td>37.4 ± 0.5</td>
<td>40.5 ± 1.2</td>
<td>38.9 ± 1.2</td>
<td>41.1 ± 1.3</td>
<td>40.2 ± 1.0*</td>
<td>41.3 ± 1.4*#</td>
<td>41.8 ± 1.6#</td>
<td>40.3 ± 1.2</td>
<td>38.2 ± 0.9</td>
<td>38.5 ± 0.8</td>
</tr>
<tr>
<td>EH</td>
<td>36.2 ± 1.1</td>
<td>41.5 ± 2.5</td>
<td>37.9 ± 2.0</td>
<td>38.3 ± 1.7</td>
<td>39.1 ± 1.9</td>
<td>37.7 ± 1.0</td>
<td>38.4 ± 0.9</td>
<td>41.7 ± 1.5*#</td>
<td>38.1 ± 1.0</td>
<td>36.9 ± 1.5</td>
</tr>
<tr>
<td>DH</td>
<td>36.6 ± 0.8</td>
<td>42.3 ± 1.8*</td>
<td>39.0 ± 1.8</td>
<td>39.3 ± 1.6</td>
<td>39.0 ± 1.4</td>
<td>38.1 ± 1.2</td>
<td>40.3 ± 1.6</td>
<td>42.0 ± 0.9*#</td>
<td>39.4 ± 1.0#</td>
<td>38.3 ± 1.8</td>
</tr>
</tbody>
</table>

Mean arterial blood pressure values (mmHg) for the sham-normothermia (SN), sham-hypothermia (SH), occlusion-normothermia (ON), occlusion-early hypothermia (EH) and occlusion-delayed hypothermia (DH) groups, 60 min before occlusion (baseline), 3, 6, 9, 12, 18, 24, 48, 72 and 168 hours after umbilical cord occlusion. Data are mean ± SEM; 96, 120 and 144 hours were not significant and have been omitted for space reasons. *p<0.05; **vs. baseline, #p<0.05; vs. sham-hypothermia.
### Table III. Electrographic Seizures.

<table>
<thead>
<tr>
<th>Group</th>
<th>Onset Seizures (h)</th>
<th>Seizure Number</th>
<th>Seizure Burden (min)</th>
<th>Duration (sec)</th>
<th>Max. Amplitude (µV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN</td>
<td>No seizures</td>
<td>No seizures</td>
<td>No seizures</td>
<td>No seizures</td>
<td>No seizures</td>
</tr>
<tr>
<td>SH</td>
<td>No seizures</td>
<td>No seizures</td>
<td>No seizures</td>
<td>No seizures</td>
<td>No seizures</td>
</tr>
<tr>
<td>ON</td>
<td>7.1 ± 0.6</td>
<td>68.1 ± 22.1</td>
<td>62.6 ± 15.3</td>
<td>60.3 ± 8.4</td>
<td>190.0 ± 27.7</td>
</tr>
<tr>
<td>EH</td>
<td>13.8 ± 4.1</td>
<td>7.0 ± 2.2*</td>
<td>3.6 ± 1.5*</td>
<td>28.3 ± 7.0*</td>
<td>122.7 ± 34.6</td>
</tr>
<tr>
<td>DH</td>
<td>13.5 ± 3.2</td>
<td>44.7 ± 28.8</td>
<td>49.0 ± 34.5</td>
<td>52.5 ± 8.7</td>
<td>127.3 ± 25.0</td>
</tr>
</tbody>
</table>

Seizure parameters for the sham-normothermia (SN), sham-hypothermia (SH), occlusion-normothermia (ON), occlusion-early hypothermia (EH) and occlusion-delayed hypothermia (DH) groups, presented as the onset time of seizures after occlusion, total number of seizures, seizure burden, seizure duration, and maximum seizure amplitude during recovery. Seizure burden was calculated as the sum duration of all seizures after occlusion. All data are presented as mean ± SEM; *p<0.05; vs. occlusion-normothermia. µV = microvolt. Note: Three fetuses in the occlusion-early hypothermia group and one fetus in the occlusion-normothermia and delayed hypothermia group did not develop seizures.
Supplemental Figures and Figure Legends

![Graphs showing EEG signals for different conditions](image-url)
Figure I. Examples of raw EEG recordings taken from individual fetuses. Sham-normothermia (SN) and sham-hypothermia (SH) animals show normal mixed high-frequency, low-amplitude EEG activity 5 hours after sham occlusion. Occlusion-normothermia (ON) shows highly abnormal epileptiform transient activity 5 hours after occlusion. Occlusion-early hypothermia (EH) shows potent suppression of abnormal epileptiform activity and partial restoration of high-frequency, low-amplitude EEG activity 5 hours after starting early hypothermia. Occlusion-delayed hypothermia (DH) shows abnormal epileptiform transient activity after starting delayed hypothermia, before the onset of delayed seizures. Note the failure of delayed hypothermia to suppress the abnormal epileptiform transient activity. µV = microvolt.
Figure II. Within subjects’ regression of 96 hourly time points after occlusion between EEG amplitude (microvolt; µV) and extradural temperature (ºC) in the sham-normothermia, sham-hypothermia, occlusion-normothermia, occlusion-early hypothermia and occlusion-delayed hypothermia groups. The lines represent individual regression lines. Different symbols represent individual animals. In the sham-normothermia group there was a significant but weak relationship between extradural temperature and EEG amplitude (0.48±1.09 µV per ºC, r=0.13, p<0.01), and similarly in the sham-hypothermia (0.22±0.24 µV per ºC; r=0.17, p<0.001) and occlusion-normothermia group (0.86±0.63 µV per ºC; r=0.19, p<0.001). Conversely, both the occlusion-hypothermia groups showed a robust relationship between extradural temperature and EEG activity, with a markedly greater suppressive effect of temperature on EEG amplitude in the occlusion-delayed hypothermia group (2.16±0.79 µV per ºC, r=0.64, p<0.001) compared to the occlusion-early hypothermia group (0.53±0.08 µV per ºC, r=0.50, p<0.001).
Figure III. Photomicrographs showing representative examples of IB4 positive microglia cells (panel A) and NeuN positive neurons (panel B) in the caudate and putamen 7 days after (sham) occlusion with normothermia or hypothermia in preterm fetal sheep. Scale bar is 200 µm.
Supplemental References


