Nonadditive Neuroprotection With Early Glutamate Receptor Blockade and Delayed Hypothermia After Asphyxia in Preterm Fetal Sheep

Sherly A. George, PhD; Robert D. Barrett, PhD; Laura Bennet, PhD; Sam Mathai, PhD; Ellen C. Jensen, PhD; Alistair J. Gunn, MBChB, PhD

Background and Purpose—Hypothermia induced after perinatal hypoxia–ischemia is partially protective. This study examined whether early treatment with the noncompetitive N-methyl-D-aspartate receptor antagonist, dizocilpine, can augment neuroprotection with delayed hypothermia after severe asphyxia in preterm fetal sheep at 0.7 weeks gestation (equivalent to 28–32 weeks in humans).

Methods—Fifty minutes after umbilical cord occlusion for 25 minutes, fetuses were randomized to either dizocilpine (2 mg/kg estimated fetal weight intravenously, then 0.07 mg/kg/h for 4 hours) and then after 5.5 hours to whole-body cooling to 3°C below baseline, or sham cooling, until 72 hours, and euthanized 7 days after umbilical cord occlusion.

Results—Delayed hypothermia was associated with improved neuronal survival (P<0.02) and reduced microglia (P=0.004) and caspase-3-positive cells (P<0.01) compared with umbilical cord occlusion. Dizocilpine was associated with reduced microglia (P<0.05) but no effect on caspase-3 induction and improved survival only in CA1/2 (P<0.05) with no apparent additive effect with delayed hypothermia.

Conclusions—Early N-methyl-D-aspartate blockade and a clinical regime of delayed whole-body hypothermia provide nonadditive neuroprotection in the preterm brain. (Stroke. 2012;43:3114-3117.)

Key Words: encephalopathy | NMDA antagonist | therapeutic hypothermia

There is increasing evidence from postmortem and imaging studies that acute subcortical neuronal injury associated in part with perinatal hypoxia–ischemia in preterm infants is an important contributor to long-term neurodevelopmental disability.1 Although hypothermia has not been evaluated in preterm infants, at term, induced mild cerebral hypothermia partially improves recovery from acute hypoxia–ischemia.2 Early anticonvulsant treatment in the P7 rat can extend the window of opportunity for delayed treatment with hypothermia.3,4 However, these studies used relatively short, less effective intervals of hypothermia. Thus, in this study, we evaluated whether early treatment with dizocilpine could augment neuroprotection with a clinical protocol of delayed, but prolonged, whole-body hypothermia after severe asphyxia in preterm fetal sheep. At this age brain development is comparable to humans at 28 to 32 weeks of gestation.5

Methods

A detailed description of methods is provided in the online-only Data Supplement.

Animal Groups and Surgery

All procedures were approved by the Animal Ethics Committee of the University of Auckland, Auckland, New Zealand. Romney/Suffolk fetal sheep (Annadale, New Zealand) were instrumented at 97 to 98 days of gestation (term=147 days), including placement of catheters for intravenous infusions and a cooling coil wrapped over the back and sides of the thorax. At 103 to 104 days gestation, fetuses received either sham umbilical cord occlusion (UCO, n=5) or UCO for 25 minutes. UCO fetuses were randomized to either UCO+vehicle (n=9), UCO+dizocilpine (Diz, n=7), UCO+hypothermia (Hypo, n=7), or UCO+Diz+Hypo (n=7).

Dizocilpine (dizocilpine hydrogenc maleate; Sigma-Aldrich Pty Ltd, Sydney, Australia) or the same volume of vehicle was given in a 2 mg/kg−1 bolus followed by a 0.07 mg/kg.h−1 constant infusion through the brachial vein from 15 minutes after UCO until 4 hours. Moderate whole body hypothermia (a 3°C reduction in esophageal temperature) was induced from 5.5 hours to 72 hours after the end of UCO by circulating cold water (10°C) through the cooling coil. After 72 hours, fetuses were allowed to rewarm over 2 hours and then euthanized 7 days after UCO.

Physiological Parameters

Plasma samples were taken during and after UCO. Fetal extradural and esophageal temperatures, mean arterial blood pressure, and...
pial electroencephalograms were recorded continuously; electroencephalographic seizures were analyzed offline.

**Histological Analysis**

Cell counts of brain sections stained for NeuN (neuronal survival), cleaved caspase-3 (a measure of apoptosis), and Isolectin B4 (IB4, activated microglia) were performed in the caudate nucleus and putamen and in the CA1/2 and CA3 regions of the hippocampus using stereological principles (online-only Data Supplement Figure I).

**Statistical Analysis**

Data were analyzed using mixed model analysis of variance followed by the Tukey post hoc test if a significant effect was found. Data are mean±SEM.

**Results**

**Blood Composition**

There were no significant differences in baseline blood gases, pH, glucose, or lactate concentrations before UCO (online-only Data Supplement Table I). UCO was associated with hypoxemia and severe mixed respiratory and metabolic acidosis, which resolved after release of UCO. Subsequently, PaO2 was higher in the UCO groups than sham controls. Hypothermia was associated with a small increase in pH and glucose levels that resolved after rewarming.

**Temperature**

Extradural and esophageal temperatures increased during dizocilpine infusion from 1 to 5 hours after UCO ($P<0.05$; online-only Data Supplement Figure II). Cooling was associated with significantly reduced temperatures from 5.5 to 72 hours ($P<0.05$).

**Mean Arterial Blood Pressure**

UCO was associated with profound hypotension followed by rebound hypertension from 1 to 2 hours compared to sham controls ($P<0.05$; online-only Data Supplement Figure II) with no subsequent between-group differences.

**Electroencephalographic Power (dB) and Electrographic Seizures**

UCO was associated with suppressed electroencephalographic power compared with sham controls from 0.5 to 72 hours after UCO ($P<0.05$) followed by progressive recovery (online-only Data Supplement Figure II), which was similar between groups. High-amplitude, low-frequency evolving seizures developed after UCO (online-only Data Supplement Table II). Delayed hypothermia and dizocilpine were associated with a significant delay before onset of seizures and reduced mean amplitude but no significant difference in the total number or duration of events.

**Neuronal Survival**

UCO was associated with severe loss of NeuN+ neurons in the striatal nuclei and the hippocampus ($P<0.001$). Delayed hypothermia was associated with improved neuronal survival in the striatum ($P=0.02$) and hippocampus ($P=0.01$; Figures 1 and 2). In contrast, dizocilpine infusion had no independent main effect in either the striatum or hippocampus ($P=0.91$ and $P=0.97$, respectively) with no additive effect. In the hippocampus, but not the striatal nuclei, there was a significant interaction of region with dizocilpine infusion ($P<0.05$), and post hoc analysis suggested significant protection with dizocilpine alone in CA1/2 (Figure 3A–B; $P<0.05$).

**Induction of Microglia and Apoptosis in Striatal Nuclei**

Delayed hypothermia was associated with reduced numbers of microglia ($P=0.004$) and caspase-3+ cells ($P<0.01$) compared with UCO. Dizocilpine infusion was associated with reduced numbers of microglia ($P<0.05$) but not caspase-3+ cells with no interaction with delayed hypothermia.

**Discussion**

Experimental hypothermia is neuroprotective if initiated as soon as possible in the first 6 hours after hypoxia–ischemia. In practice, cooling is typically initiated 4 to 4.5 hours after birth. It is likely that this delay is a major factor underlying the modest clinical improvement in normal survival.
has been considerable interest in whether early drug therapy could extend the window of opportunity for treatment with hypothermia.3,4 In the present study, mild whole-body cooling in preterm fetal sheep, delayed by 5.5 hours and continued for 72 hours, was associated with partial neuroprotection. Although there was a striking improvement in neuronal survival in CA1/2, to near sham control values, CA3 and striatal nuclei were only partially protected. This improvement was associated with marked suppression of the inflammatory response to asphyxia and reduced cleaved caspase-3 expression.

In contrast, initiation of N-methyl-D-aspartate receptor blockade from 15 minutes after asphyxia was independently neuroprotective, but only in the CA1/2 region of the hippocampus. This was a more modest effect than previously reported after hypoxia–ischemia in neonatal rodents and preterm fetal sheep.5,6,9 The dizocilpine dose in the present study was titrated to compensate for transplacental passage and was associated with electroencephalographic suppression and delayed onset of seizures. Intriguingly, microglial activation was suppressed, consistent with evidence that activated microglia express the N-methyl-D-aspartate receptor and, in vitro, posthypoxic dizocilpine reduces microglial activation and expression of inflammatory factors.10

The finding that early drug treatment did not augment protection with delayed hypothermia contrasts with previous findings that treatment with anticonvulsants such as topiramate and phenobarbital started 15 minutes after hypoxia-ischemia in P7 rats synergistically increased neuroprotection with hypothermia started up to 3 hours later.3,4 It is possible that the contrasting findings may reflect differences in species, insult, or receptor-specific effects. Furthermore, we used a longer interval of delayed hypothermia, consistent with clinical protocols,7 which may have reduced the scope to demonstrate improvement with combined therapy. Earlier and mildly deeper cooling would be more protective and, given the increase in brain temperature with dizocilpine,7 might improve synergism, but at the cost of reduced scope to show combined effects.

In conclusion, these data suggest that early N-methyl-D-aspartate blockade and a clinical regime of delayed whole-body hypothermia provide nonadditive neuroprotection.

Source of Funding

This study was funded by the Health Research Council of New Zealand. Dr Barrett received the William Georgetti Scholarship.
Figure 3. Neuronal cell counts (A) and photomicrographs (B) showing neuronal loss in the hippocampus. *P<0.05 versus sham occlusion, #P<0.05 versus UCO. Plates (B): left column, NeuN+ cell counts in the CA1–2 region of hippocampus. Right column, NeuN+ cell counts in CA3 region of hippocampus. Scale bar=75 μm. UCO indicates umbilical cord occlusion.

Disclosures
None.

References
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The University of Auckland, Auckland, New Zealand.
Supplementary Methods

Experimental preparation

All procedures were approved by the Animal Ethics Committee of The University of Auckland, New Zealand. Romney/Suffolk fetal sheep were instrumented using sterile techniques at 97-98 days (d) of gestation (term = 147 d). Food, but not water was withdrawn 18 hours (h) before surgery. Ewes were given 5 ml of Streptocin (procaine penicillin (250,000 IU) and dihydrostreptomycin (250 mg.ml\(^{-1}\)), Stockguard Labs Ltd, Hamilton, NZ) intramuscularly for prophylaxis 30 minutes (min) prior to the start of surgery. Anaesthesia was induced by intravenous (i.v.) injection of Alfaxan (Alphaxalone, 3 mg.kg\(^{-1}\), Jurox, Rutherford, AU), and general anaesthesia maintained using 2-3% halothane in O\(_2\). Ewes were not ventilated and the depth of anaesthesia, maternal heart rate and respiration were constantly monitored by trained anesthetic staff. Under anesthesia a 20 gauge catheter was placed in a maternal front leg vein for the duration of surgery, and the ewes were placed on a constant infusion of isotonic saline drip (250 ml.h\(^{-1}\)) to maintain maternal fluid balance.

All surgical procedures were performed using sterile techniques.\(^1\) Catheters were placed in the left fetal femoral artery and vein, the right brachial artery and vein and the amniotic sac. Two pairs of electroencephalogram (EEG) electrodes (AS633-7SSF, Cooner Wire Co., Chatsworth, USA) were placed on the dura over the parasagittal parietal cortex (5 mm and 10 mm anterior to bregma and 5 mm lateral) and secured with cyanoacrylate glue (Super Glue, Selleys, Auckland, NZ). A reference electrode was sewn over the occiput. A thermistor (Incu-Temp-1, Mallinckrodt Medical Inc., St. Louis, USA) was placed over the parasagittal dura 20 mm anterior to bregma, and a second thermistor placed deep in the esophagus. An inflatable silicone occluder was also placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, USA). The fetus was returned to the uterus and all fetal leads were exteriorized through the maternal flank. The maternal long saphenous vein was also catheterized to provide access for post-operative maternal care and euthanasia. Antibiotics (80 mg Gentamicin, Pharmacia and Upjohn, Perth, AU) were administered into the amniotic sac prior to closure of the uterus.

Following surgery, sheep were housed together in separate metabolic cages with access to water and food ad libitum. They were kept in a temperature-controlled room (16 ± 1°C, humidity 50 ± 10%), in a 12 h light/dark cycle. A period of 4-5 d post-operative recovery was allowed before experiments were commenced, during which time antibiotics were administered daily for 4 d i.v. to the ewe (600 mg Benzylpencillin Sodium, Novartis Ltd., Auckland, NZ; 80 mg Gentamicin). Fetal arterial blood was taken daily from the brachial artery for blood gas analysis for the assessment of fetal health. Catheters were maintained patent by continuous infusion of heparinized isotonic saline (20 U.ml\(^{-1}\) at 0.2 ml.h\(^{-1}\)).

Experimental Design and Recordings

Experiments were conducted at 103-104 d gestation. Fetal extradural and esophageal temperatures and physiological data were recorded continuously from 12 h before UCO until 7 days (168 h) afterwards. Signals were averaged at 1 min intervals and stored on disk by custom software (Labview for Windows, National Instruments Inc., Austin, USA). The EEG signal was low-pass filtered at 30 Hz and the intensity spectrum extracted. For data presentation, the total EEG intensity (power) was normalized by log transformation (dB, 20 × log (intensity)), and data from left and right EEG electrodes averaged to give mean total EEG intensity.

Umbilical Cord Occlusion (UCO)

Fetuses received either sham UCO (n=5) or UCO, induced by rapid inflation of the umbilical cord occluder for 25 min with isotonic saline of a defined volume known to completely inflate the occluder. Successful occlusion was confirmed by observation of a rapid fall in fetal heart and suppression of the EEG in all cases. Occlusion fetuses were randomized to either UCO+vehicle (n=9), UCO+dizocilpine (Diz) (n=7), UCO+hypothermia (Hypo) (n=7), or UCO+Diz+Hypo (n=7).

Dizocilpine (dizocilpine hydrogen maleate, Sigma-Aldrich Pty. Ltd., Sydney, AU) was dissolved in sterile isotonic saline at a concentration of 1 mg.ml\(^{-1}\). Dizocilpine or the same volume of vehicle was given in a 2
mg.kg⁻¹ bolus followed by a 0.07 mg.kg.h⁻¹ constant infusion through the brachial vein from 15 min following the end of UCO until 4 h.

Moderate whole body hypothermia was induced from 5.5 h to 72 h after the end of UCO by circulating cold water (10°C) through a cooling coil made of silicon tubing, 1.02 mm x 2.15 mm (Bamford & Co Ltd, Wellington, NZ), wrapped around the back and sides of the fetal body. Cooling was titrated in the first 2 h to reduce fetal extradural temperature from 39.4 ± 0.1°C to between 36 and 37°C (i.e. a 3°C reduction). After 72 h fetuses were allowed to rewarm by increasing the circulating water temperature in steps over 2 h. At 7 days after the end of UCO 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).

**Histological Analysis**

Fetal brains were perfusion-fixed *in situ* with 0.9% saline solution followed by 500 ml of 10% phosphate-buffered formalin. Following removal from the skull, tissue was fixed for a further 3 d and embedded using a standard paraffin tissue preparation.¹

**Immunohistochemistry**

As previously described,¹² for NeuN and cleaved caspase-3 immunohistochemistry, the rehydrated/antigen retrieved sections were incubated with 2.5% normal horse serum (NHS) for NeuN, or 2.5% normal goat serum (NGS) for caspase-3, for 1 h at room temperature (RT), followed by mouse anti-NeuN (Chemicon International Inc., Temecula, CA, USA; 1:400), or rabbit anti-Cleaved Caspase 3 (Cell Signaling Technology, MA, USA; 1:200) in PBS plus 2.5% NHS or NGS for one night at 4°C. Biotin-conjugated secondary antibody (1:200 in PBS overnight at 4°C), avidin-biotin complex (1:50, 2 h at RT; Vectastain Elite ABC Kit, Vector Laboratories), and 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, Sydney, AU) were used to visualize the positive signal. For control sections the primary antibody was omitted from incubation solution. Staining was performed in separate batches, with each batch containing 4 sections from each animal from all experimental groups.

**Isolectin B4 (IB4) Staining**

The rehydrated/antigen retrieved sections were incubated with biotinylated Isolectin B4 (Sigma-Aldrich Pty. Ltd., Sydney, AU; diluted 1:100 in PBS-T) overnight at 4°C. Sections were then washed 3 x 5 min in PBS-T, incubated in avidin-biotin complex (VectorStain Elite ABC Kit, Vector Laboratories, Burgingame, USA) in the ratio of 1:50 in PBS for 2 h at room temperature, washed in PBS (3 x 5 min), and then visualized using diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich Pty, Ltd.). The reaction was terminated by washing in distilled water, the sections dehydrated and mounted. Control sections were processed in the same way except that the Isolectin B4 stain was omitted from the incubation solution (negative control).

**Neuron, Microglia and Caspase 3 positive Cell Counts.**

For each animal, three (hippocampus) and four (striatum) adjacent 8 µm serial sections of the forebrain were analyzed from 14 to 17 mm anterior to stereotaxic zero (level of the dorsal horn of the anterior hippocampus, and the mid-striatum). Neurons in the hippocampus, and neurons, IB4+, and caspase 3+ cells in the striatum were counted using Stereoinvestigator software (version 8; Micro-BrightField, Williston, VT) and light microscopy (Nikon [Tokyo, Japan] eclipse 80i; Scitech, Preston, Victoria, Australia) 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 2x magnification, and then randomly translating a grid onto the sections and applying a fractionator probe consisting of a counting frame for object inclusion/exclusion at 40x magnification. The grid and counting frame sizes for the CA1/2 and CA3 regions were 200 x 150 µm and 50 x 50 µm, respectively. The grid and counting frame sizes used for the caudate nucleus 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. Cell counts for the hippocampus for each region were converted to density (cells/mm²) by the following formula: [Total Markers Counted / (Number of Sampling Sites x Counting Frame Area)] x 10⁶. For the caudate nucleus and putamen, the estimated total by mean measured thickness (4 slides cut at 8 µm
thickness, with 1 slide every 8 sampled giving a total thickness of 164 µm), was calculated using the Stereoinvestigator software. Final density for each region of interest was calculated by averaging results from the three adjacent serial sections.

**Data analysis**

The raw EEG was visually assessed for overt electrographic seizures, defined as the concurrent appearance of sudden, repetitive, evolving stereotyped waveforms, lasting more than 10 seconds, with an amplitude greater than 20 µV.\(^1\) Long-term recovery data were calculated as 1 h averages from end of UCO until 24 h, and thereafter as 6 h averages until 168 h. The baseline period was taken as the mean of the 6 h before UCO.

The effect of treatment on EEG intensity, blood gas variables, and cell counts were evaluated using mixed model ANOVA including independent effects of hypothermia or dizocilpine and with time or subregion as a repeated measure (SPSS v12, SPSS Inc., Chicago, IL, USA) followed by the Tukey post-hoc test when a significant overall effect was found. Between-group comparisons for seizure-related parameters were made using the independent samples Kruskal-Wallis test. Statistical differences were considered significant at \(P<0.05\). Data are presented as mean ± standard error (SEM).
Table S1. Physiological parameters taken from fetal blood samples. S: Sham, UCO; umbilical cord occlusion, UDH; UCO plus dizocilpine plus hypothermia, UH; UCO plus hypothermia, UD; UCO plus dizocilpine.

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| UDH | 1.1±0.1 | 0.4±0.1** | 0.7±0.1** | 1.5±0.2 | 1.3±0.1 | 1.7±0.1 | 1.3±0.2 | 1.6±0.1 | 1.6±0.1* | 1.7±0.1;base1 # | 1.6±0.1*#
|     | 1.1±0.3 | 0.4±0.1** | 0.8±0.1** | 1.6±0.3 | 1.2±0.2 | 1.4±0.3 | 1.3±0.2 | 1.2±0.2 | 1.8±0.2* | 1.5±0.2# | 1.6±0.5# |
|     | 1.0±0.1 | 0.3±0.1** | 0.5±0.1** | 1.4±0.2 | 1.2±0.1 | 1.4±0.1 | 1.3±0.1 | 1.3±0.1 | 1.4±0.2 | 1.1±0.1& | 1.2±0.1 | 1.1±0.1 |

**P<0.001, vs. sham controls; * P<0.05, vs. sham controls; # P<0.05, vs. UCO; $ P<0.05, UCO+Hypo vs. UCO+Diz; & P<0.05, UCO+Diz vs. UCO+Diz+Hypo; @ P<0.05, UCO+Hypo vs. UCO+Diz+Hypo.
Table S2: Characteristics of post-asphyxial seizures

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* p<0.05 vs UCO
Supplementary Figure S1. Photomicrographs of coronal sections of a near-term fetal sheep brain showing the fields used for analysis. A, Caudate and putamen. B, CA1-2 and CA3 regions of Hippocampus. Scale bar (A and B) = 2.5 mm
Supplementary Figure S2. Time sequence of changes in fetal temperature, EEG power, and mean arterial blood pressure (MAP) in the 5 experimental groups. Note the increase in extradural temperature from 1-5 h after UCO during dizocilpine infusion ($P<0.05$). Dashed line, $P<0.05$, both dizocilpine groups compared to the sham group. Black bar, $P<0.05$, both hypothermia groups compared to the sham group. EEG power was significantly reduced in
the dizocilpine and hypothermia growth compared to UCO alone during the infusion and cooling phases respectively, but there was no significant difference between groups in the final 48h of recordings. There was no significant increase in MAP at baseline between any of the groups. There was no significant effect of hypothermia or dizocilpine on MAP throughout the experimental period compared to the sham group.

References


2. Bennet L, Roelfsema V, George S, Dean JM, Emerald BS, Gunn AJ. The effect of cerebral hypothermia on white and grey matter injury induced by severe hypoxia in preterm fetal sheep. J. Physiol. 2007;578:491-506