Spontaneous pre-existing hypoxia does not affect brain damage after global cerebral ischaemia in late-gestation fetal sheep

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Joanne Davidson, Laura Bennet and Alistair J. Gunn conceptualized and designed the study. Caroline Yuill, Guido Wassink, Joanne Davidson undertook experiments and analysed data. Caroline Yuill completed the histological analysis and prepared the histological figures. All authors critically reviewed the manuscript and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Abstract

There is considerable evidence that a mild, non-injurious insult can protect (precondition) against a subsequent injurious insult. Typically protection is seen when the gap between insults is several days to a week. However, the effect of mild but persistent hypoxia is unknown. In this study we examined the hypothesis that mild pre-existing hypoxia (PaO_2 < 17 mmHg) would reduce neural injury in chronically instrumented late-gestation (0.85 gestation) fetal sheep exposed to 30 min of global cerebral ischaemia induced by bilateral carotid artery occlusion (normoxia: n = 9 vs pre-existing hypoxia: n = 9) or normoxia plus sham ischaemia (sham controls: n = 9). Histopathology was assessed after seven days recovery. Fetuses with pre-existing hypoxia had lower PaO_2 values (16.1 ± 0.6 vs 26.0 ± 1.1 mmHg) and were lighter at post-mortem (4033 ± 412 vs 5261 ± 238 g) compared to normoxic fetuses. Cerebral ischaemia was associated with secondary cortical oedema and seizures, reduced final EEG power, loss of sleep state cycling and significant loss of neurons and oligodendrocytes, with no significant effect of pre-existing hypoxia. Pre-existing hypoxia was associated with a significantly attenuated rise in mean arterial pressure between 18 and 36 hours and slower resolution of cortical oedema between 96 and 150 hours after ischaemia. These data suggest that chronic hypoxia is not associated with a significant preconditioning effect.

Introduction

There is strong preclinical evidence that a non-injurious period of mild hypoxia or brief ischaemia can protect (precondition) against or sensitise to a subsequent injurious insult such as prolonged hypoxia-ischaemia or ischaemia in the brain and the heart [1-4]. Preconditioning is highly dependent on the timing of the mild insult. Consistent protection has been reported after 3 to 4 hours of mild to moderate hypoxia given 24 hours before severe hypoxia-ischaemia in neonatal rodents [5-10] and piglets [11]. However, these studies examined discrete events that are followed by a period of normoxic recovery before the severe insult. It is not clear whether mild but chronic hypoxia as may occur before birth is also protective.

Spontaneous chronic hypoxia can occur in late gestation, for example related to placental compromise, and can be associated with intrauterine growth restriction (IUGR), greater risk of stillbirth and poor long-term neurodevelopmental outcomes [12,13]. Experimentally, mild pre-existing hypoxia in near-term fetal sheep has been associated with more rapid chemoreflex adaptation to prolonged umbilical cord occlusion but no effect on fetal arterial blood pressure during occlusion [14]. In contrast, at a similar age, severe pre-existing chronic hypoxia (average PaO_2 11.4 ± 0.9 mmHg vs 21.4± 0.6 mmHg in normoxic animals) followed by exposure to repeated brief asphyxia at a rate consistent with early labour, was associated with hypotension, cephalic hypoperfusion, greater EEG suppression, inter-occlusion seizures and more sustained cytotoxic brain oedema, consistent with an earlier onset of neural injury [2]. These contrasting outcomes may reflect differences in cardiac tolerance, potentially related to reduced glycogen stores [15].

In the present study, we examined whether pre-existing spontaneous mild hypoxia (defined as PaO_2 < 17 mmHg 24 hours or 48 hours before ischaemia) would improve outcome from

subsequent exposure to 30 minutes of global cerebral ischaemia in the near-term fetal sheep, at a gestational age when neural maturity is broadly similar to the full term infant [16]. An ischaemic insult was used to avoid confounding related to the cardiac response to asphyxia. Recovery of brain activity, seizures and return of sleep state cycling were assessed by EEG recordings and cortical cytotoxic oedema by changes in cortical impedance [17]. Changes in cardiovascular physiology were assessed by continuous recording of mean arterial pressure (MAP), fetal heart rate (FHR) and carotid artery blood flow (CBF). Neuronal and oligodendrocyte cell survival were quantified using immunohistochemistry.

Methods

Fetal surgery

All procedures were approved by the Animal Ethics Committee of The University of Auckland. In brief, 27 time-mated Romney/Suffolk fetal sheep were instrumented using sterile technique at 118-124 days gestation (term is 145 days). Food, but not water was withdrawn 18 hours before surgery. Ewes were given 5 mL of Streptocin (procaine penicillin (250,000 IU/mL) and dihydrostreptomycin (250 mg/mL, Stockguard Labs Ltd, Hamilton, New Zealand)) intramuscularly for prophylaxis 30 minutes before surgery. Anaesthesia was induced by I.V. injection of Alfaxan (alphaxalone, 3 mg/kg, Jurox, Rutherford, New South Wales, Australia), and general anaesthesia maintained using 2-3% isoflurane in O_2. The depth of anaesthesia, maternal heart rate and respiration were constantly monitored by trained anaesthetic staff. Ewes received a constant infusion of isotonic saline (0.2 mL/h) to maintain fluid balance.

Following a maternal midline abdominal incision and exteriorisation of the fetus, both fetal brachial arteries were catheterised with polyvinyl catheters to measure mean arterial blood pressure. An amniotic catheter to allow for correction of pressures with maternal movement was secured to the fetal shoulder. ECG electrodes (Cooner Wire Co., Chatsworth, California, USA) were sewn across the fetal chest to record fetal heart rate. The vertebral-occipital anastomoses were ligated and inflatable carotid occluder cuffs were placed around both carotid arteries [18,19]. A 3S Transonic ultrasonic flow probe (Transonic systems, Ithaca, NY) was placed around the right carotid artery. Using a 7 stranded stainless steel wire, two pairs of EEG electrodes (AS633–7SSF; Cooner Wire Co.) were placed on the dura over the parasagittal parietal cortex (10 mm and 20 mm anterior to bregma and 10 mm lateral) and secured with cyanoacrylate glue. A reference electrode was sewn over the occiput. To

measure cortical impedance a third pair of electrodes was placed over the dura 5 mm lateral to the EEG electrodes. A further two electrodes were sewn in the nuchal muscle to record electromyographic activity as a measure of fetal movement. A thermistor was placed over the parasagittal dura 30 mm anterior to bregma. The uterus was then closed and antibiotics (80 mg gentamicin, Pharmacia and Upjohn, Rydalmere, New South Wales, Australia) were administered into the amniotic sac. The maternal laparotomy skin incision was repaired and infiltrated with a local analgesic, 10 ml 0.5% bupivacaine plus adrenaline (AstraZeneca Ltd., Auckland, New Zealand). All fetal catheters and leads were exteriorized through the maternal flank. The maternal long saphenous vein was catheterised to provide access for post-operative maternal care and euthanasia.

Post-operative care

Sheep were housed together in separate metabolic cages with access to food and water *ad libitum*. They were kept in a temperature-controlled room (16 ± 1°C, humidity 50 ± 10%), in a 12 hour light/dark cycle. Antibiotics were administered daily for four days I.V. to the ewe (600 mg benzylpencillin sodium, Novartis Ltd, Auckland, New Zealand, and 80 mg gentamicin). Fetal catheters were maintained patent by continuous infusion of heparinized saline (20 U/mL at 0.15 mL/h) and the maternal catheter maintained by daily flushing.

Data recording

Data recordings began 24 hours before the start of ischaemia and continued for the remainder of the experiment. Data were recorded and saved continuously to disk for off-line analysis using custom data acquisition programs (LabView for Windows, National Instruments Ltd., Austin, Texas, USA). Arterial blood samples were taken for pre-ductal pH, blood gas, base excess (Ciba-Corning Diagnostics 845 blood gas analyser and co-oximeter, Massachusetts, USA), glucose and lactate measurements (YSI model 2300, Yellow Springs, Ohio, USA).

Experimental protocols

Experiments were performed at 128 ± 1 d gestation. Normoxia was defined as fetal PaO_2 > 17 mmHg for all days after surgery and before ischaemia. Pre-existing hypoxia was defined as PaO_2 < 17 mmHg within 48 hours before ischaemia. Ischaemia was induced by reversible inflation of the carotid occluder cuffs with saline for 30 minutes. Successful occlusion of the carotid arteries was confirmed by rapid suppression of EEG activity. Fetuses were allowed to recover for seven days and ewes were then killed with an overdose of sodium pentobarbitone (9 g I.V. to the ewe; Chemstock International, Christchurch, N.Z.).

Data analysis

Off-line analysis of the physiological data was performed using customised Labview programmes. Seizure burden was analysed visually by a masked observer from raw EEG activity as the duration of seizures per hour for the first 48 hours after the end of ischaemia. Seizures were classified as stereotypic evolving epileptiform activity greater than 20 μV in amplitude and longer than 10 seconds in duration. Sleep state cycling was defined on the one minute averaged EEG data as a repetitive alternating pattern of high and low voltage activity, with each phase lasting approximately 20 minutes. The day on which sleep state cycling resumed was determined visually by a masked observer. Animals that did not resume sleep state cycling by the end of the experiment were assigned a value of seven days. As these data were not normally distributed, a Mann-Whitney test with the Bonferroni correction was used for statistical analysis. Other data were analysed by ANOVA or repeated measures ANOVA with time as a repeated measure as appropriate, followed by the Tukey post-hoc test when a significant difference was found. Statistical significance was accepted when $p < 0.05$.

Immunohistochemistry

The fetal brains were perfusion fixed with 10% phosphate-buffered formalin. Slices (10 µm thick) were cut using a microtome (Leica Jung RM2035, Solms Germany) starting at the level where the dorsal hippocampus became apparent. Two slides per animal per stain were assessed. Slides were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Slides were washed in 0.1 mol/L phosphate buffered saline (PBS). Antigen retrieval was performed using citrate buffer in an antigen retrieval system (EMS Antigen 200 Retriever, Emgrid, Australia) followed by incubation in 1 % H_20_2 in methanol for NeuN and PBS for olig2. Blocking was performed in 3.5% normal goat serum (NGS) for 1 h at room temperature. Sections were labelled with 1:400 mouse anti-neuronal nuclei monoclonal antibody (NeuN, Chemicon International, Temecula, CA, USA) or 1:400 Olig2 (Chemicon International) overnight at 4°C. Sections were incubated in biotin-conjugated secondary 1:200 anti-mouse IgG (for NeuN) or 1:200 anti-rabbit IgG (for Olig2, a marker for oligodendrocytes at all stages of the lineage [20]) in 3.5% NGS. Slides were then incubated with ExtrAvidin® (1:200, Sigma-Aldrich Pty. Ltd, St Louis, USA) in PBS for two hours at room temperature and then reacted in diaminobenzidine tetrachloride (Sigma-Aldrich Pty. Ltd.). The reaction was stopped by washing in PBS and the sections were dehydrated and mounted.

Photomicrographs were imaged at 40x magnification using light microscopy (Nikon eclipse 80i, Scitech Ltd, Preston, Victoria, Australia) and the surviving cells counted using image J, by an investigator masked to the treatment group. NeuN-labelled neurons were assessed according to morphological characteristics and were excluded if cells showed a condensed nucleus or fragmented appearance [21]. Neurons were assessed in two regions of the parasagittal cortex, and the CA1, CA3, CA4 and the dentate gyrus of the hippocampus. Olig2-labelled surviving oligodendrocytes were quantified in the intragyral white matter of the first parasagittal gyrus and the periventricular white matter.

Results

Biochemistry and physiology

Baseline PaO_2 values were lower in the pre-existing hypoxia group than the normoxia group and remained significantly lower at all time-points throughout the experiment ($p < 0.05$). There were no significant differences in baseline pH, $PaCO_2$, glucose and lactate measurements between the normoxia and pre-existing hypoxia groups (Table 1), and no significant differences between groups for any physiological parameters during the baseline period. After ischaemia, $PaCO_2$ was significantly reduced at day one in the pre-existing hypoxia group and significantly increased from baseline at day seven in the normoxia group ($p < 0.05$). Lactate was similarly elevated from baseline at day one in both the normoxia and pre-existing hypoxia groups ($p < 0.05$). Glucose values were significantly elevated from four hours after ischaemia until day one in the normoxia group and on day one in the pre-existing hypoxia group ($p < 0.05$). A transient increase in mean arterial pressure was seen in the normoxia group between 18 and 36 hours, which was significantly attenuated in the pre-existing hypoxia group (Figure 1, $p < 0.05$). No significant differences were seen between the pre-existing hypoxia and normoxia groups for carotid artery blood flow, fetal heart rate, nuchal EMG or extradural temperature at any time.

Electrophysiology

Ischaemia was associated with a rapid reduction in EEG power and spectral edge frequency to below baseline levels, followed by a rapid increase in cortical impedance in both ischaemia groups (Figures 2 and 3), compared to sham controls ($p < 0.001$, data not shown). After ischaemia EEG power and spectral edge frequency remained below baseline in the normoxia group, and cortical impedance resolved nearly to baseline values over the first hour.

EEG power transiently increased from 9 to 48 hours after ischaemia, corresponding to intense seizure activity in both the normoxia and pre-existing hypoxia groups. Cerebral impedance increased secondarily between 12 and 48 hours (Figure 2 and Figure 3). There were no significant differences in the recovery of EEG activity or spectral edge frequency although there was significantly slower resolution of cytotoxic oedema between 96 and 150 hours after the end of ischaemia in the pre-existing hypoxia group compared to the normoxia group. There were no significant differences in seizure burden between the normoxia and pre-existing hypoxia groups (Figure 3). Similarly, there was no significant difference in the time taken for return of sleep state cycling (normoxia: 5 ± 0.6 days *vs*. pre-existing hypoxia: 4 ± 0 days). 3/9 fetuses in the normoxia group and 2/9 fetuses in the pre-existing hypoxia group did not resume clearly defined sleep state cycling by the end of day seven.

Immunohistochemistry

Both ischaemia groups showed significantly lower numbers of neurons in the parasagittal cortex and CA1, CA3, CA4 and dentate gyrus of the hippocampus seven days after ischaemia compared to the sham control group ($p < 0.05$, Figure 4). There was no significant difference in neuronal number in any region between the pre-existing hypoxia and normoxia groups. Similarly, there was a significant reduction in oligodendrocytes in the intragyral and periventricular white matter seven days after ischaemia compared to sham controls ($p < 0.05$, Figure 5), with no significant difference between the normoxia and pre-existing hypoxia groups.

Post-mortem

The pre-existing hypoxia group were lighter than the normoxia group at post-mortem ($p < 0.05$, Table 2), and tended to have reduced body weight compared to sham controls ($p = 0.07$). There was a similar sex distribution between all groups. One normoxic fetus was a

twin compared to three twins in the pre-existing hypoxia group (N.S.). Both the normoxia and pre-existing hypoxia groups had reduced post-mortem brain weights compared to sham controls ($p < 0.05$).

Discussion

This study demonstrates that mild pre-existing hypoxia was not associated with any material preconditioning effect against 30 min of global cerebral ischaemia in near-term fetal sheep. There was no significant effect of mild pre-existing hypoxia on burden of seizures or recovery of EEG power, spectral edge frequency or return of sleep state cycling, or survival of neurons and oligodendrocytes, or brain weight seven days after ischaemia.

Preconditioning has been consistently shown experimentally 24 hours after mild to moderate hypoxia in rodents and piglets [5-11]. These findings of course are related to discrete events, whereas in the present study, stable, mild hypoxia was present for 24 hours or more before cerebral ischaemia, well within the typical interval required to induce preconditioning [22]. Given that fetal body weight was less in the pre-existing hypoxia group, it is very likely that relative placental restriction was present compared to normoxic animals.

Despite this evidence for long-lasting hypoxia, there was no evidence for neuroprotection in fetuses with pre-existing hypoxia. Global cerebral ischaemia was associated with suppression of EEG power in both the normoxia and pre-existing hypoxia groups, which remained suppressed until the onset of seizures, and secondary cell swelling as measured by a rise in cortical impedance [17]. There was no significant difference in the timing or extent of the secondary rise in cortical impedance between the normoxia and pre-existing hypoxia groups. There was no difference in the EEG amplitude, spectral edge frequency or seizure burden, nor the carotid artery blood flow, fetal heart rate or nuchal EMG during the period of seizure activity between groups. However, the late fall in cortical impedance after the resolution of seizure activity to below baseline and sham control values was slightly, although significantly, slower in the pre-existing hypoxia group. Previous studies have shown that there is markedly increased cerebral blood flow and intracerebral blood volume during this

phase [23,24]. Reducing this secondary hyperperfusion with inhibition of nitric oxide synthase attenuated the hyperperfusion, but increased cerebral injury [25]. These data suggest that greater microcirculation is beneficial, perhaps by improved supply of micronutrients to moderately injured regions. Thus, speculatively, the delayed fall in impedance may reflect reduced oxygen delivery to such regions.

Final EEG power remained below baseline levels with sleep state cycling taking between four and five days to recover, with three animals in the normoxia and two animals in the pre-existing hypoxia failing to return to sleep state cycling within the recovery period. Clinically, in normothermic infants with moderate to severe HIE, lack of recovery of sleep wake cycling within the first 36 to 48 h of life is associated with a significantly worse outcome [26,27]. Consistent with this, histologically, the severe neuronal loss in the parasagittal cortex and the CA1, CA3, CA4 and dentate gyrus of the hippocampus and of oligodendrocytes in the periventricular and intragyral white matter after ischaemia [28,29], was not affected by pre-existing hypoxia. There was significant attenuation in the rise in mean arterial pressure that was associated with the period of seizure activity. Given that there was no significant difference in seizure burden or body movements associated with seizures, potentially the attenuated rise in mean arterial pressure may have been related to altered cardiac or cardiovascular reflex responses.

There is growing clinical evidence in adults that transient ischaemic attacks (TIA) can protect against later ischaemic stroke [30,31]. Moncayo *et al* [30] showed that patients without prior TIAs had a more severe clinical picture on admission and a greater reduction in consciousness, whereas patients with previous TIAs had a more favourable outcome. In addition, they suggested that there may be a critical duration of TIA for ischaemic preconditioning, as shown by better outcomes in patients with TIAs lasting for 10 to 20 minutes. Further, less benefit was seen in patients that had only one previous TIA or who had

more than three previous TIAs. The highest proportion of favourable outcomes was seen in patients with an interval of one week or less between the TIAs and the cerebral infarct, supporting the hypothesis that preconditioning has a finite duration [30].

This hypothesis is highly consistent with experimental data showing that the duration of preconditioning is limited in time. For example, in a classical study in adult gerbils, a noninjurious period of ischaemia, induced by 2 min bilateral carotid artery occlusion reduced damage from a subsequent 3 min bilateral carotid artery occlusion, provided that the preconditioning insult was applied at least 24 hours and no more than seven days before the more severe insult [32]. This temporal evolution is likely related to the evolving induction of genes such as hypoxia inducible factor (HIF-1α) [4]. Although HIF-1α appears to be an important mediator, since preconditioning does not occur in HIF-1α knockout mice, it is important to appreciate that over 1000 genes are induced within the first 24 hours after hypoxia [33]. Thus, the lack of a preconditioning effect in the current study may be due to the chronic nature of the pre-existing hypoxia, such that any potential protective mechanisms may have resolved before the study.

Alternatively, it is possible that ongoing hypoxia may have inhibited upregulation of neuroprotective genes. In the human as well as in the sheep, IUGR is generally a consequence of placental compromise [12,13]. In the sheep, such placental compromise can occur as a result of conditions such as heat stress and over nutrition in adolescent ewes [34,35]. In addition, inflammatory markers are seen in a subset of children who develop cerebral palsy, both with and without concomitant IUGR [36]; in turn such inflammation may influence the response to acute cerebral hypoxia-ischaemia [37]. We do not know specifically whether inflammation occurred in the cases of spontaneous hypoxia in the present study, but there is evidence that IUGR induced by single umbilical artery ligation in the fetal sheep is

associated with persistent inflammation [38]. Further studies would be important to dissect the effects of hypoxia and placental inflammation in IUGR.

The present study cannot rule out the possibility that a discrete, limited period of hypoxia might be protective, as suggested by rodent studies. However, the more common clinical setting is chronic hypoxia in growth restricted fetuses, and exposure to severe hypoxia-ischaemia around birth [36]. Importantly, the present study shows that there was no clear protective or deleterious effect of mild spontaneous pre-existing hypoxia on global cerebral ischaemia. A limitation of this study was the use of a spontaneously occurring period of pre-existing hypoxia. Both the extent and duration of the pre-existing hypoxia may have contributed to variability within this group. However, such periods of spontaneous chronic hypoxia can occur clinically and therefore it is important to understand how they may affect the development of ischaemic brain injury in the fetus. In conclusion, the present study suggests that mild pre-existing hypoxia is not associated with material neuroprotection against severe cerebral ischaemia compared with normoxia in near-term fetal sheep.

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Table 1. Blood gas, pH, glucose and lactate data before and after 30 min of global cerebral ischaemia in the pre-existing hypoxia and normoxia groups.

pH	**Baseline**	**2h**	**4h**	**6h**	**1d**	**7d**
Normoxia	7.4±0.01	7.39±0.01	7.41±0.01	7.40±0.01	7.38±0.02	7.36±0.01
Pre-existing hypoxia	7.37±0.01	7.35±0.02	7.37±0.01	7.38±0.01	7.35±0.02	7.35±0.01
$PCO_{2\ (mmHg)}$	**Baseline**	**2h**	**4h**	**6h**	**1d**	**7d**
Normoxia	44.6±1.4	42.6± 1.6	43.4±1.4	44.9±1.9	44.4±2.7	49.4±1.9†
Pre-existing hypoxia	49.2±1.0	47.4±0.5	47.1±0.7	48.1±0.6	45.5±1.3#	49.4±1.5
$PO_{2\ (mmHg)\ *}$	**Baseline***	**2h ***	**4h ***	**6h ***	**1d ***	**7d***
Normoxia	26.0±1.1	25.8±1.2	25.9±1.3	25.4±1.9	25.0±2.0	23.0±1.1†
Pre-existing hypoxia	16.1±0.6	18.0±1.0	18.4±0.8#	18.6±0.8#	16.0±0.8	18.4±0.9
Lactate (mmol/L)	**Baseline**	**2h**	**4h**	**6h**	**1d**	**7d**
Normoxia	1.1±0.1	2.4±0.5	2.3±0.5	2.0±0.4	4.7±1.1†	1.2±0.1
Pre-existing hypoxia	1.3±0.2	2.4±0.5	1.9±0.4	1.6±0.3	3.2±0.4#	1.0±0.1
Glucose (mmol/L)	**Baseline**	**2h**	**4h**	**6h**	**1d**	**7d**
Normoxia	1.0±0.1	1.3±0.1†	1.3±0.1†	1.3±0.1†	1.5±0.1†	1±0.1
Pre-existing hypoxia	0.7±0.1	1.0±0.1	1.0±0.1	0.9±0.1	1.1±0.1#	0.6±0.1

*$p<0.05$ pre-existing hypoxia versus normoxia groups, ɨ Normoxia group versus baseline, # pre-existing hypoxia group versus baseline. Data are mean±SEM. Days 2-6 have been omitted as there were no significant differences between groups.

Table 2. Post-mortem body and brain weight, fetal sex in the sham control, normoxia and pre-existing hypoxia groups seven days after 30 min of global cerebral ischaemia.

Group	Body weight (g)	Brain weight (g)	Sex (M / F)
Sham control	4892±290	50.6± 1.7	5 / 4
Normoxia	5261± 238	44.0± 1.5*	5 / 5
Pre-existing hypoxia	4033.4± 412#	45.7± 1.5*	3 / 5 / 1U

M = male, F = female, U = unknown. *$p<0.05$ versus sham control, #$p<0.05$ versus normoxia group.

Figure legends

Figure 1. Changes in carotid artery blood flow (CaBF), nuchal EMG, fetal heart rate (FHR) and extradural temperature before, during and after 30 min of global cerebral ischaemia in the normoxia and pre-existing hypoxia groups. There were no significant differences in carotid artery blood flow, nuchal EMG, fetal heart rate and extradural temperature. There was a significant attenuation in the rise in mean arterial pressure between 18 and 36 hours in the pre-existing hypoxia group compared to the normoxia group ($p<0.05$).

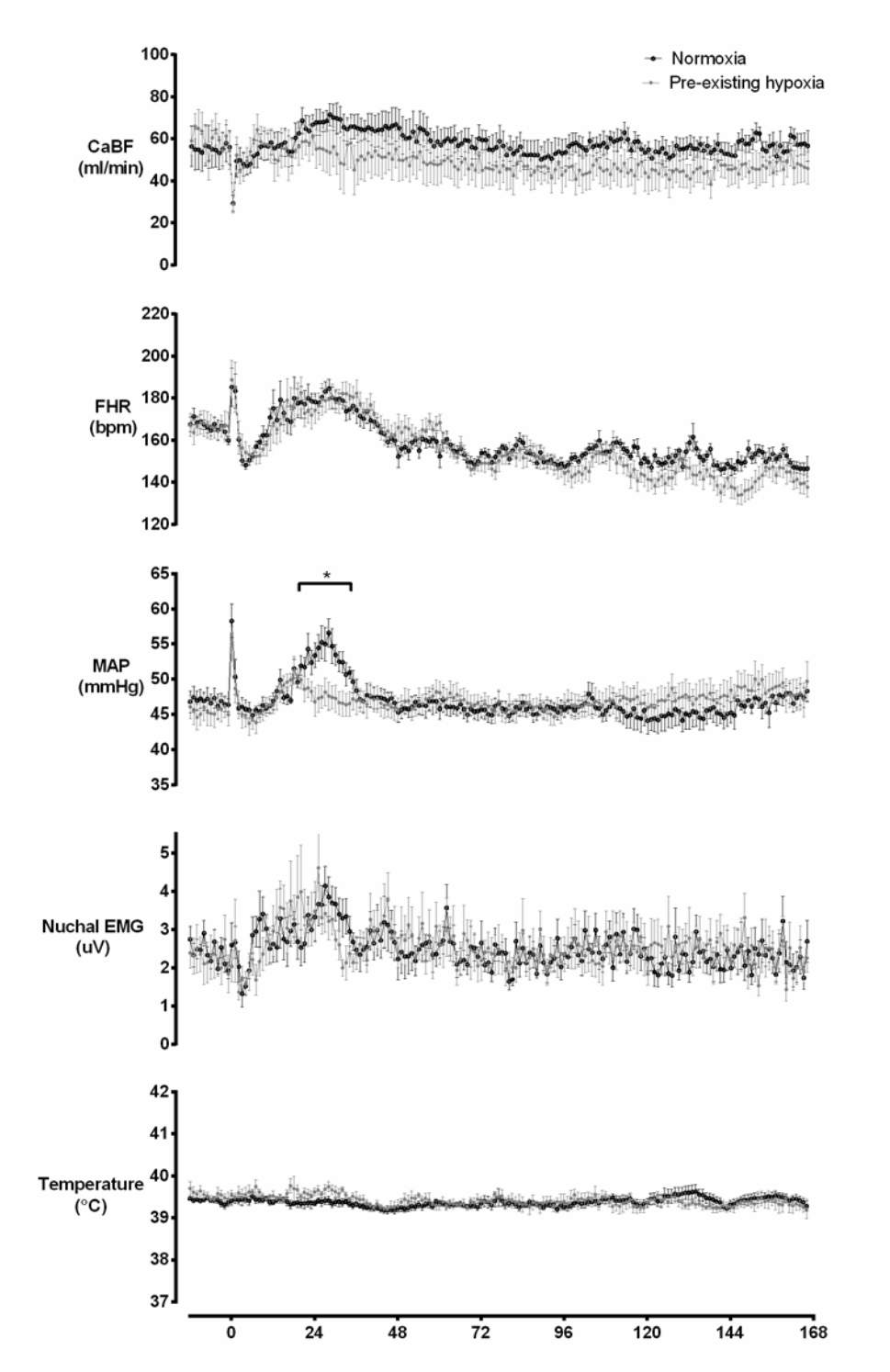
Figure 2. Changes in EEG power, spectral edge frequency and cortical impedance before, during and after 30 min of global cerebral ischaemia in the normoxia and pre-existing hypoxia groups. There were no significant differences in the recovery of EEG activity or spectral edge frequency although there was a significantly slower fall in cytotoxic oedema as measured by cerebral impedance between 96 and 150 hours after the end of ischaemia ($p<0.05$).

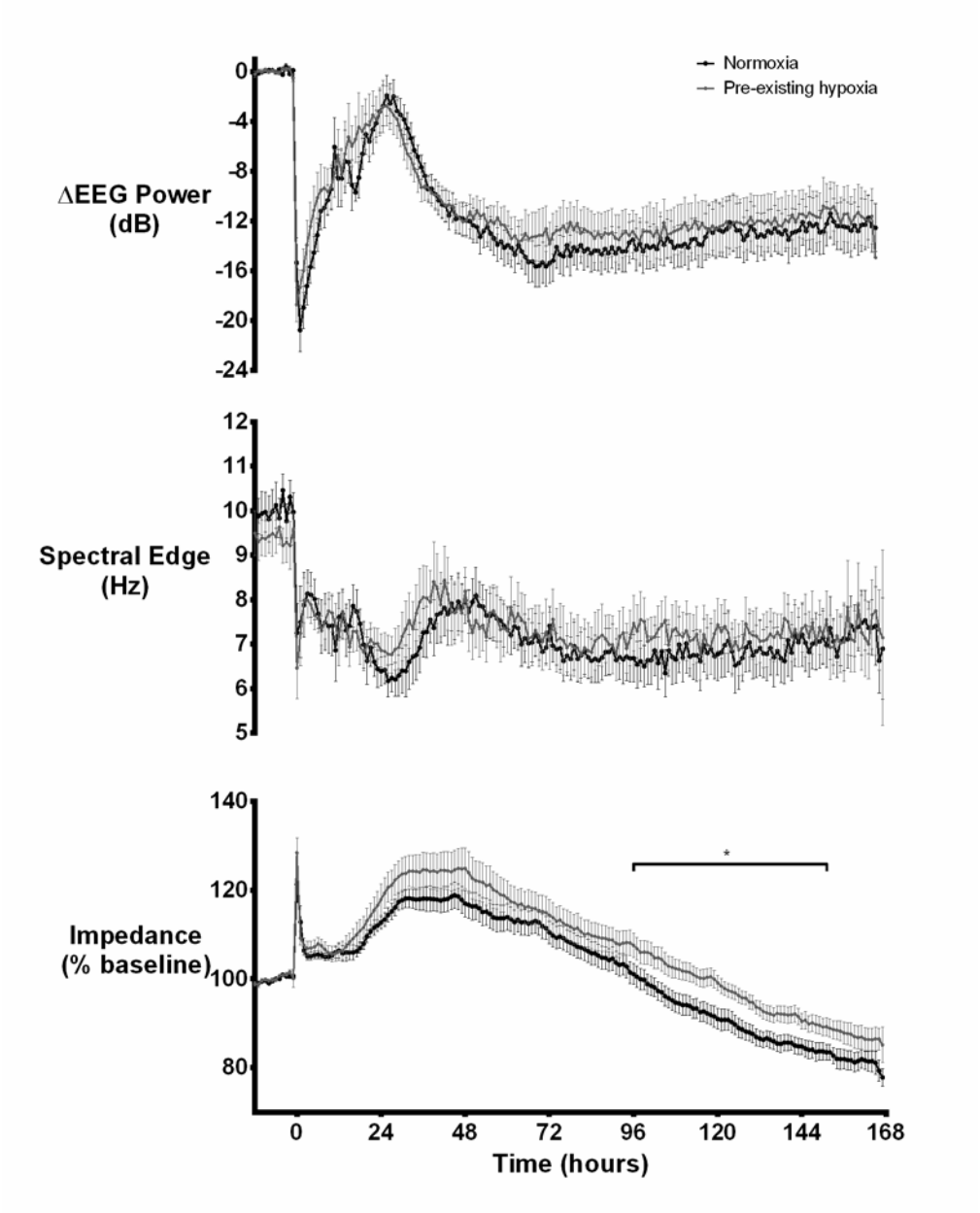
Figure 3. Seizure burden quantified as time spent having seizures per hour in the normoxia and pre-existing hypoxia groups after 30 min of global cerebral ischaemia. There was no significant difference in seizure burden in the 48 hours after the end of ischaemia between the normoxia and pre-existing hypoxia groups ($p> 0.05$).

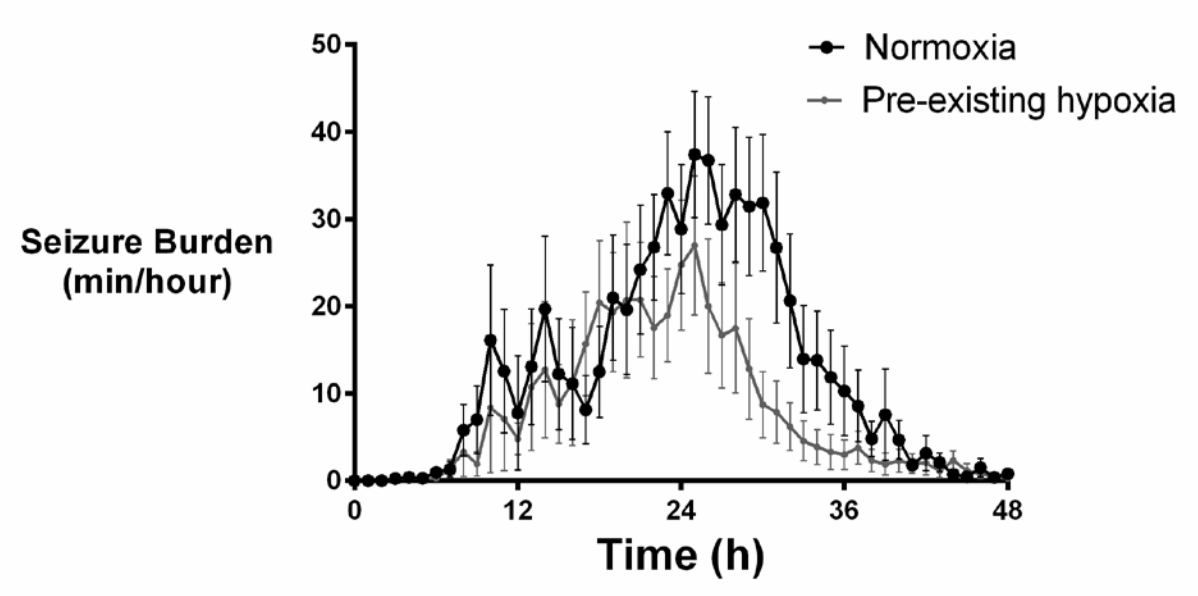
Figure 4. Neuronal survival in the cortex and hippocampus seven days after 30 min of global cerebral ischaemia in the normoxia and pre-existing hypoxia groups. Top panel: A significant reduction in neuronal survival was seen in the parasagittal cortex and the CA1, CA3, CA4 and dentate gyrus of the hippocampus in both the normoxia and pre-existing hypoxia groups compared to sham control ($p<0.05$), with no significant difference between the normoxia and pre-existing hypoxia groups in any area ($p> 0.05$). Bottom panel: Photomicrographs showing NeuN-positive neuronal survival in the sham control (A-E), normoxia (F-J) and pre-existing

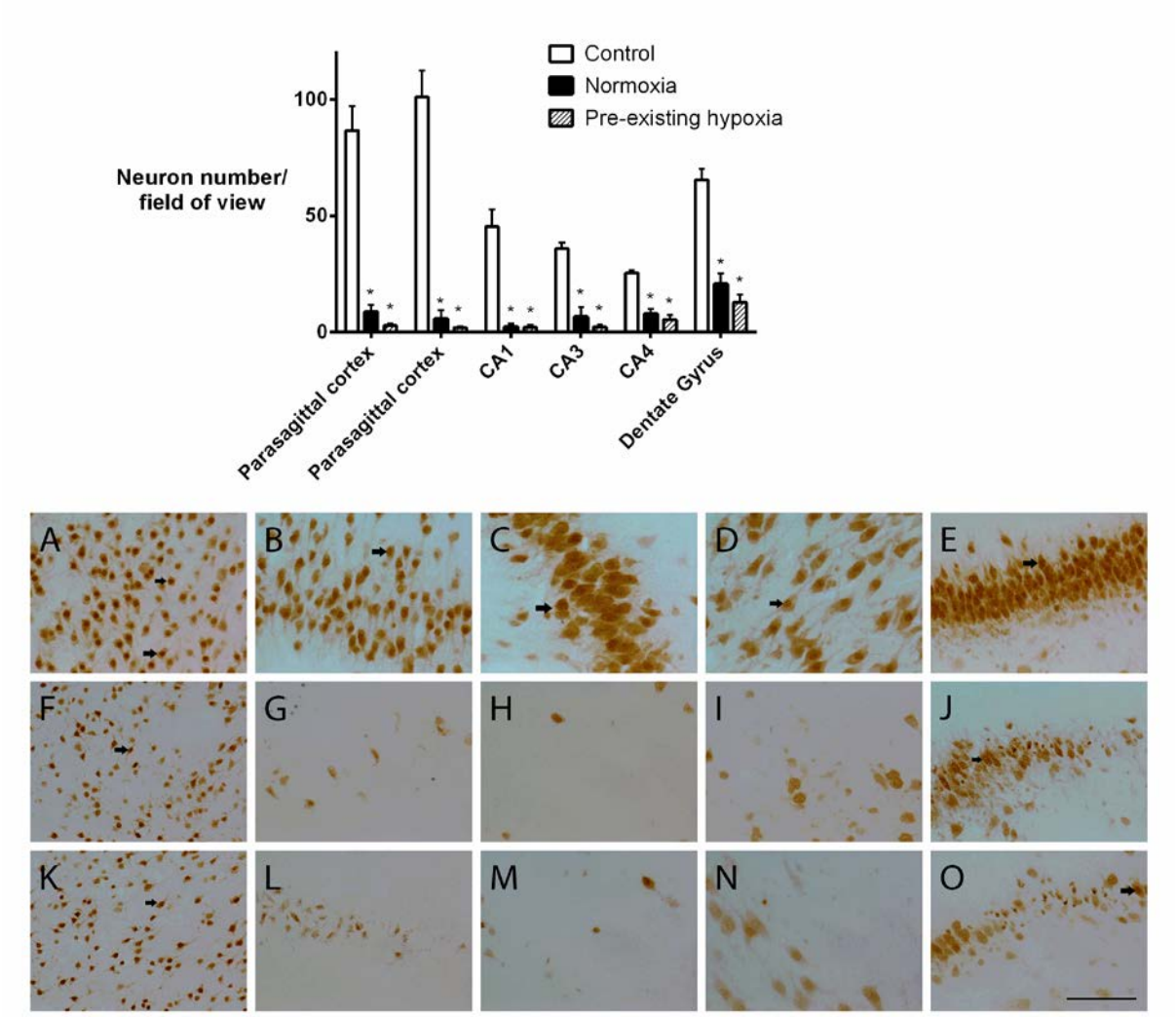
hypoxia (K-O) groups in the parasagittal cortex (A, F, K), CA1 (B, G, L), CA3 (C, H, M), CA4 (D, I, N) and dentate gyrus (E, J, O). Arrows indicate examples of healthy neurons. Scale bar 100 μm.

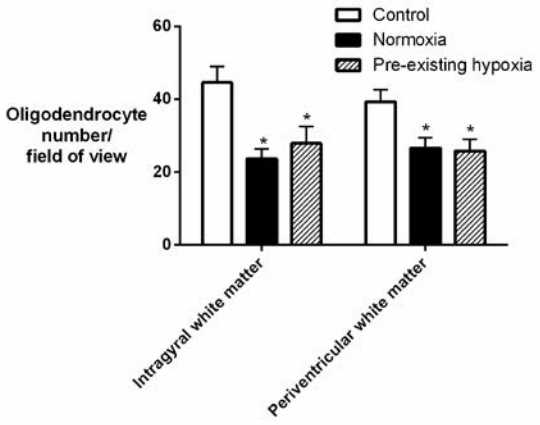
Figure 5. Oligodendrocyte survival in the intragyral and periventricular white matter seven days after 30 min of global cerebral ischaemia in the normoxia and pre-existing hypoxia groups. Left panel: There was a significant reduction in oligodendrocyte survival in both the normoxia and pre-existing hypoxia groups in the intragyral and periventricular white matter compared to the sham control group ($p<0.05$). There was no significant difference between the normoxia and pre-existing hypoxia groups for neuron or oligodendrocyte number in any area ($p> 0.05$). Right panel: Photomicrographs showing Olig-2 positive oligodendrocytes in the sham control (A,B), normoxia (C,D) and pre-existing hypoxia (E,F) groups in the intragyral (A,C, E) and periventricular (B,D,F) white matter. Arrows indicate examples of healthy oligodendrocytes. Scale bar 100 μm.

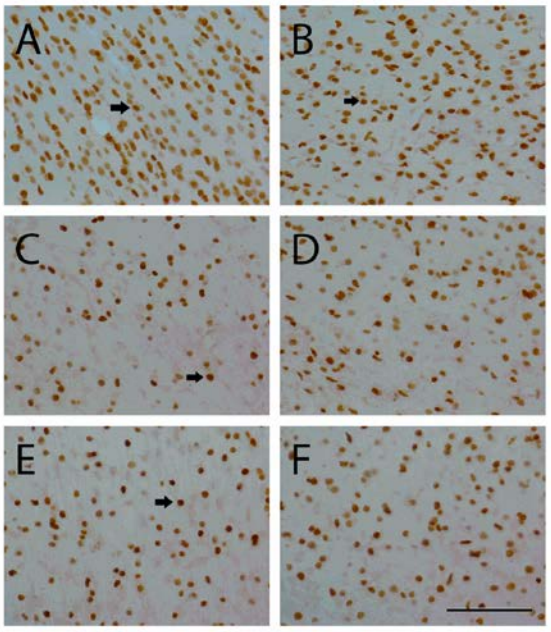

Normoxia
Pre-existing hypoxia
CaBF (ml/min)
100
80
60
40
20
0
FHR (bpm)
220
200
180
160
140
120
MAP (mmHg)
65
60
55
50
45
40
35
*
Nuchal EMG (uV)
5
4
3
2
1
0
Temperature (°C)
42
41
40
39
38
37
0
24
48
72
96
120
144
168


Normoxia
Pre-existing hypoxia
ΔEEG Power (dB)
0
-4
-8
-12
-16
-20
-24
Spectral Edge (Hz)
12
11
10
9
8
7
6
5
Impedance (% baseline)
140
120
100
80
*
0
24
48
72
96
120
144
168
Time (hours)


Seizure Burden
(min/hour)
50
40
30
20
10
0
0
12
24
36
48
Time (h)
Normoxia
Pre-existing hypoxia


Control
Normoxia
Pre-existing hypoxia
Neuron number/
field of view
100
50
0
Parasagittal cortex
Parasagittal cortex
CA1
CA3
CA4
Dentate Gyrus
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O


Control
Normoxia
Pre-existing hypoxia
Oligodendrocyte number/ field of view
60
40
20
0
*
*
*
*
Intragyral white matter
Periventricular white matter


A
B
C
D
E
F