Oxygen And Its Effects On The Developing Brain

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Declaration

The following work is original and completed by myself as part of studies towards this MD thesis.

I contributed significantly to the setting up and running of the rodent experiments. I did all my own tissue harvest, sample processing and analysis for the rodent work. For the MRA project, Katharine Josephs, a BMedSci student that I was supervising at the time, completed the analysis.

The samples used for the human study had previously been collected as part of the Scottish Perinatal Neuropathology study. I identified the samples to be used for this study, prepared the sections for and performed the immunohistochemistry. I did all the visual and computer analysis, apart from that clearly identified as being the work of Professor Jeanne Bell.

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Abstract

Survival following preterm birth is improving but with an associated increase in morbidity in the survivors in the form of learning difficulties, attention, dyspraxia and dyslexia.

Retinopathy of prematurity is a disease of disordered vascularisation within the retina. One of the most significant risk factors for developing this disease is the use of postnatal oxygen. Within Edinburgh this has been examined in more detail in a rodent model of prematurity. Previous work has shown that these rat pups experience white matter injury when subjected to physiological variable oxygen. The following body of work looks at white matter injury in more detail.

The vascularity within the rodent brain has been assessed both through histological study and magnetic resonance angiography (MRA). We hypothesise that the same disease process that is seen in the retinas of these infants is also occurring within the white matter of the brain. Rat pups reared in a variable oxygen appear to have more cerebral capillaries, which have an increased diameter when compared to rat pups reared in room air. Assessment by MRA shows that the larger cerebral vessels have a smaller volume in pups reared in oxygen when compared to room air.

The human study was set up to look for differences in pathology seen between brains from stillborn infants and those who experienced a neonatal death. It has been shown that there appears to be a greater astrocytic response in brains from neonatal deaths when compared to stillbirths but no difference in expression of myelin basic protein (MBP). It has been shown that quantity of MBP expression increases in relation to an increase in gestational age.

This body of work contributes to the discussions regarding what is deemed to be a safe level of oxygen to use within the neonatal unit.

Abbreviations

ACA	Anterior cerebral artery
ADHD	Attention deficit hyperactivity disorder
CI	Confidence interval
COX	Cyclooxygenase
DAB	Diaminobenzidine
DEHSI	Diffuse excessive high signal intensity
DF	Distance factor
DQ	Developmental quotient
ELBW	Extremely low birth weight
FITC	Fluorescein isothiocyanate
GFAP	Glial Fibrillary Acidic Protein
HIF 1α	Hypoxia inducible factor
ICA	Internal carotid artery
INOS	Inducible nitric oxide synthase
IUGR	Intrauterine growth restriction
MBP	Myelin Basic Protein
MCA	Middle cerebral artery
MMP	Matrix metalloproteinase
MRA	Magnetic Resonance Angiography
NF-κB	Nuclear Factor kappa B
NO	Nitric Oxide
NSS	Normal swine serum
OPC	Oligodendrocyte precursor cell
OR	Odds ratio
PBS	Phosphate buffered saline
PECAM 1	Platelet endothelial adhesion molecule
PFA	Paraformaldehyde
PGE2	Prostoglandin E2
PGF2α	Prostoglandin F2α
PP	Predictive probablity
PSN	Pontosubicular necrosis
PVL	Periventricular Leukomalacia
RF	Radiofrequency
ROP	Retinopathy of prematurity
SOD	Superoxide dismutase
TBS	Tris buffered saline
TE	Excitation time
TGF	Transforming growth factor
TNF-α	Tumour necrosis factor alpha
TOF	Time of flight
TR	Relaxation time
TXA 2	Thromboxane
VEGF	Vascular endothelial growth factor
VLBW	Very low birth weight
VWF	Von Willebrand's factor

INTRODUCTION

Oxygen And Its Role In Pathology

Oxygen

Oxygen is essential for life and has been known about since the 18th century. It appeared to do nothing but good in the treatment of the sick. Since the 1930s it has been used widely in the care of newborn infants.

Oxygen is essential in the respiratory chain within the mitochondria of cells. It is needed for the process of oxidative phosphorylation, which generates ATP from molecules of glucose producing energy for life.

$$C_6H_2O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + 30\ ATP$$

This process is tightly controlled to minimise the production of partially reduced oxygen and thus free radicals. Around 95-98% of the oxygen in a cell is used by the respiratory chain[1].

Free Radicals

Free radicals were first described in the 1960s. They are highly reactive and, as such, were initially thought to have no role in the normal biological processes. This concept was re-evaluated with the discovery of an antioxidant, superoxide dismutase, and it is now accepted that free radicals have a role in many biological processes. Their effects are controlled by various antioxidants. Part of the damage caused by reperfusion following ischaemia is caused by a surge in the amount of oxygen available to the tissues and these antioxidant processes becoming saturated[2].

Oxygen is usually converted into the superoxide free radical within the respiratory chain but its levels are restricted by the presence of antioxidants. Free radicals are also produced by the enzyme xanthine oxidase. This is present in endothelial cells within the blood brain barrier making this a target for injury. Ischemic injury leads to an increase in the amount of hypoxanthine present, which reacts with the excess of oxygen that is available following reperfusion and creates the superoxide radical[3]. Free radicals are also produced by NADPH oxidase, which is present in many other cerebral tissues including neurons and phagocytes such as microglia.

Superoxide can be converted into hydrogen peroxide, which in excess, is also damaging. This can react with free iron within the mitochondria via the Fenton reaction to produce hydroxyl radicals.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Hydoxyl radicals react with lipid peroxides to produce alkoxyl radicals. These cause damage by increasing microvascular permeability, oedema, inflammation and cell death. They alter membrane function by interfering with receptors, enzymes and ion channels[2,3,4].

Nitric oxide (NO) was discovered in the 1990s and is an intra- and extra-cellular signalling molecule. It is produced by the enzyme nitric oxide synthase, which is present in three isoforms: neuronal, endothelial and inducible. NO has both beneficial and detrimental properties. It is a potent vasodilator and is useful in maintaining cerebral and retinal circulation; however, in excess it exacerbates neuronal injury as it has pro-apoptotic properties[5].

Fernandez studied NO within the cerebral cortex of rats and showed an up regulation of neuronal nitric oxide synthase in the presence of hypoxia. This initially contributed to restoration of blood flow to the area but also triggered free radical cascades[6]. NO is one of the mediators that is involved in the process of angiogenesis through the angiogenic factor called Vascular Endothelial Growth Factor (VEGF). It has been shown to mediate endothelial cell proliferation via VEGF. The structural organisation of endothelial cells by VEGF is conducted in a NO dependent manner. Short term exposure to VEGF appears to increase NO production and longer term exposure results in an increase in the enzyme endothelial nitric oxide synthase [7].

Nitric oxide is not the only free radical involved in the regulation of angiogenesis or vascular tone. Superoxide initially causes vascular relaxation but then in increased concentration leads to arterial / arteriolar constriction[5]. This role in regulating vascular tone along with other free radicals such as nitric oxide explains some of the link between free radicals and vasculature related disease processes such as retinopathy of prematurity.

Nitric oxide alone is a harmful substance but combined with superoxide it forms peroxynitrite in the fastest biological reaction known. Peroxynitrite is the molecule that results in most of the damage[3].

$$O_2^{\cdot -} + {}^{\cdot}NO^{-} \longrightarrow OO\text{-}N{=}O$$

Superoxide + nitric oxide → peroxynitrite

This reaction is more favourable than the antioxidant reaction involving superoxide dismutase. Peroxynitrite is also damaging because of its ability to cross lipid membranes, which enables it to cause damage away from its site of production. As a result it is 400 times more damaging than superoxide itself.

Peroxynitrite causes its damage through a variety of mechanisms. Primarily it damages DNA and inhibits DNA repair. As a result it causes protein oxidative damage, formation of protein carbonyls, lipid peroxidation, oxidation of thiols, nitration of proteins and hydroxylation of phenolics. This results in a variety of problems. It causes damage to the mitochondria disrupting ATP synthesis and induces cell death. It can also induce apoptosis by the activation of caspases[8].

Protection Against Free Radical Damage

The damage caused by free radicals is reduced by the presence of a range of antioxidants. They are essential in the normal homeostasis of the body.

Superoxide dismutase (SOD) is an antioxidant enzyme that transforms superoxide into oxygen and hydrogen peroxide. It is present throughout the body and probably represents around 0.5% of total brain weight. It is up regulated at times of injury. In animal studies administration of SOD has been shown to reduce the size of cerebral infarcts by up to 35%[9].

Glutathione is present within the mitochondria of the cell and is important in antioxidant processes. In reacting with superoxide it is transformed into its reduced form. The amount of reduced glutathione present can be measured as a marker of oxidative damage. Other antioxidants include vitamin A and E, and bilirubin. Problems are encountered when these defences are overwhelmed or are too immature to function[10].

Oxygen Environment In Utero

In utero the arterial oxygen pressure is around 20 mmHg. At the time of birth all infants are subjected to a rapid increase in partial pressure of oxygen up to50mmHg. The term infant is ready to adapt to this and has functional antioxidant systems in place[11]

The preterm infant is less able to manage free radicals. Their antioxidant systems are immature, as they are not needed in utero. They are especially deficient in glutathione. This results in an excess of free radicals and the opportunity for damage to occur. The preterm brain is particularly susceptible due to its high concentration of fatty acids that are vulnerable to lipid peroxidation by free radicals[10].

Work has been done to assess the fetal oxygen saturation levels during labour. The fetal saturations were found to be 58% +/- 10% in normal labour. As a result it is not surprising that it can take between 5 and 15 minutes for a healthy term infant to establish oxygen saturations of >93%[12]. This data can not necessarily be extrapolated to the preterm infant as they are rarely delivered as a result of an acute asphyxial event and so need more stabilisation rather than resuscitation at birth. But it does provide evidence that newborn infants do not need to have their oxygen saturations optimised by administration of excess oxygen in the first few minutes of life.

Oxygen At Resuscitation

Oxygen has been used in neonatal resuscitation since 1780. At this time it was administered by use of bellows. This practice was discredited and not revisited until the mid 1800s. Positive pressure ventilation was invented in America in 1928 and in the UK in 1935, this was combined with endotracheal intubation. Resuscitation of infants in 60% oxygen in an air lock was tried during the 1950s. This air lock administered increases in pressure to simulate the contracting uterus in an effort to help the infant adapt to extra-uterine life. Initial studies showed it made no difference to the babies' outcomes and a subsequent study claimed it was as effective as intubation and positive pressure ventilation. Techniques of administering oxygen have been refined but how much should be administered to these babies is unknown[13].

The 2010 Resuscitation Council guidelines state that resuscitation of term newborns with room air initially is preferable to using oxygen[14]. There is evidence to suggest that babies recover better following resuscitation with room air when compared to resuscitation in 100% oxygen[15,16].They take less time to their first cry and less time to establish a regular respiratory pattern. There is also evidence suggesting that resuscitation with room air results in a 5% reduction in neonatal mortality[16]. The guidelines also provide a table of recommended oxygen saturation levels to be aimed for during resuscitation. These values have been taken from two observational studies that studied oxygen saturations in the first ten minutes of life in infants who were deemed to not need resuscitation, as they had a heart rate greater than 100/min and were breathing regularly[17,18].

In preterm infants there is less definitive guidance regarding whether resuscitation should be started in room air or oxygen. The Dawson study concluded that preterm infants obtained "normal" saturation levels at a slower rate than term infants. They acknowledged though that the optimal saturation target for a preterm infant is still to be defined[18]. Wang et al performed a study with 41 infants less than 32 weeks and randomised them to being resuscitated with room air or 90% oxygen. They had a well-defined protocol as to when it was appropriate to increase the amount of oxygen being used during the resuscitation. Their aim was to obtain saturation levels of 80-85% by 5 minutes. They showed that all infants in the room air group needed some oxygen for rescue therapy to reach the 5 minute saturation target. The conclusion of this study was that it was not safe to start resuscitation in preterm infants in room air[19].

Escrig et al have also randomised preterm infants to be resuscitated in either low or high oxygen concentrations. They chose to use 30% oxygen as a start point as they acknowledged the failure of other studies to show that room air was effective in these cohorts. Their high oxygen group received 90% oxygen. Their target saturation was 85% by 10 minutes of life. They concluded that infants stabilised sooner when resuscitated with room air and obtained the same saturation levels as those resuscitated with higher levels of oxygen. When the data is scrutinised more carefully though it shows that many of the infants actually required transient increases in inhaled oxygen due to failure to reach target heart rates or saturations. Infants in the room air group did receive less oxygen overall than those in the high oxygen group[20].

Babies resuscitated in 100% oxygen show evidence of oxidative stress with both reduced levels of glutathione and increased levels of superoxide dismutase present at the time of the event[21].This effect is still present after four weeks[15], although it is unclear if this has any effect on long-term outcome. Follow-up data from babies resuscitated with either room air or 100% oxygen has failed to demonstrate any neurodevelopmental differences in children at 18-24 months. This can be interpreted to mean that resuscitation with room air is not to the detriment of the children's development. Additionally, it could be concluded that the oxidative damage seen in the infants in the first month of life has no long term effect on their neurodevelopment[22].

There is concern that even transient hyperoxia, as experienced in a resuscitation attempt, may cause alterations in cerebral blood flow that may adversely affect outcome. Rootwelt et al investigated this in pigs rendered hypoxic by ventilation with 8% oxygen and then resuscitated either with 21% or 100% oxygen. The pigs were injected with micro spheres labelled with a radio-nuclide, which was removed at a constant rate. From this, calculations relating to blood flow could be made from the relative concentration of radio-nuclide in post mortem specimens. They could not find any difference between cerebral blood flow between the groups[23]. Contradicting this finding though, Solas et al found that cerebral blood flow was restored to normal levels more quickly in pigs resuscitated with 100% oxygen as opposed to room air in most areas of the brain. They also demonstrated an improved response in mean arterial blood pressure[24].

Bagenholm et al performed a simple study assessing brain weights as a marker of neurological injury in rats resuscitated with either room air or 100% oxygen. They found no difference in weights and concluded that there was no difference in cerebral injury between the groups. This is a very crude measure of assessing brain injury[25].

In adult mice, resuscitation with 100% oxygen results in an increase in NF-κB (nuclear factor kappaB) levels in the brain when compared to those resuscitated with room air. NF-κB is an important factor in the inflammatory response, where an increase in levels implies an increase in cellular damage. This mouse model is not the most relevant to infant resuscitation in view of the age of the animals, but the results should not be ignored[26].

Piglets subjected to hypoxia show an increased amount of free radical damage following resuscitation with 100% oxygen using glycerol as a marker of brain damage. Glycerol was found to be 50% higher in the oxygen-treated group when compared to room air. A reduced level of antioxidants, and an increase in MMP-2 (matrix metallo proteinases) and MMP-3 was found in the piglet brains. These matrix metalloproteinases are implicated in ischaemic injury[27].

There is evidence to suggest that short-term neurological outcome is improved following resuscitation with room air as opposed to 100% oxygen. Piglets were subjected to a pneumothorax induced hypoxic insult and then resuscitated with either room air or 100% oxygen. There was no demonstrable difference in markers of oxidative stress or histopathology between the groups. The hyperoxic group were, however, more neurologically depressed at four hours after the event[28].

A recent review article concluded that there is no definitive answer as to what amount of oxygen should be used to resuscitate a preterm infant but that it would be reasonable to commence in 30-40% oxygen and then titrate the amount of oxygen used with saturation monitoring results[29]. Practices vary between hospitals and according to the availability of equipment to deliver variable concentrations of inspired oxygen.

Use Of Oxygen On The Neonatal Unit

Use of oxygen on the neonatal unit has decreased since the association between high inspired oxygen and retinopathy of prematurity was noted. With the widespread use of surfactant and an increased understanding in the physiology of preterm infants there is a trend to using even less oxygen within the NNU. In spite of this it can not be avoided completely and even use of small amounts is not without its problems.

Hyperoxia results in an increased ventilatory rate and an increased metabolic rate in term infants. Whilst on the neonatal unit, transient hyperoxia is associated with alterations in cerebral blood flow[30]. Increasing a premature infant's oxygen levels to three times the resting amount results in a reduction in blood flow velocity. This does not normalise immediately on return to normoxia. The effect is even greater in term infants but they also experience a corresponding drop in CO_2 levels, which on further analysis has been shown to have the greater impact on blood flow[31]. A reduction in cerebral blood flow has been associated with an increase in peri-ventricular leukomalacia and germinal matrix haemorrhage. However, there are several other factors that need to be considered before this can be accepted as a direct association. In this study by Pryds there was, unfortunately, no information regarding oxygen extraction and cerebral metabolic rate[32].

Retinopathy Of Prematurity (ROP)

In 1951 Campbell et al suggested there was a link between use of oxygen in the neonatal unit and the incidence of retinopathy of prematurity. This theory was tested in the laboratory using extremes of hyperoxia (>80%) and it was concluded definitively that oxygen had a significant role in disease causation. Since that time neonatologists have been increasingly cautious about the use of oxygen in the treatment of premature babies[33]. More evidence is accumulating as to what is deemed to be a "safe" level of oxygen saturation.

In Edinburgh in 1995 it was established that ROP was present even in infants who had been cared for in minimal levels of hyperoxia. The risk of development of ROP was greatest if the exposure to oxygen occurred within the first 14 days of life. It was also concluded that rather than it being the actual level of hyperoxia that dictated disease severity, it was the variability in transcutaneous oxygen monitoring that appeared to be more important. The babies who were more stable had less ROP[34].

In utero the retina develops normally in a relatively hypoxic environment (2-3 kPa). When an infant is born prematurely they are thrown into a relatively hyperoxic environment even when maintained in only minimal hyperoxia. As a consequence their antioxidant defences are overwhelmed and they lack the ability to handle this excess in oxygen. As a result the process of vascularisation of the retina becomes disorganised and leads to ROP. In brief the hyperoxia results in a reduction of hypoxia inducible factor secretion and therefore a reduction in vascular endothelial growth factor secretion. This leads to there being a reduction in the amount of vessel formation within the retina. Eventually the periphery of the retina becomes hypoxic as its metabolic demands outstrip supply. This hypoxia produces an up-regulation in HIF 1α and therefore VEGF, leading to an uncontrolled increase in vessel

development. Unfortunately this surge of activity is quite disorganised and results in the pathology of ROP[35].

Oxygen Saturation Targets

Oxygen saturation targets in the newborn period are a subject of ongoing debate and research[36,37]. A term newborn baby that does not require resuscitation has initial saturation levels from 43-77%. By five minutes the mean level is only 89%[38]. Saturation monitoring at the time of resuscitation is beset with practical problems mainly related to motion artefact. Other problems encountered include poor perfusion, vernix, acrocyanosis and high ambient light[39].

Oxygen saturation levels in a preterm infant are less well studied. Kamlin et al showed that it took healthy preterm infants, without the need for resuscitation, significantly longer to achieve saturation levels of >90% when compared with term infants – 6.5minutes vs 4.7 minutes[40].

Kopotic and Lindner used pulse oximetry in the resuscitation room to aid management decisions in the resuscitation of 50 infants. In this was a subgroup of 15 preterm infants. The infants received 100% oxygen as standard practice during resuscitation attempts. They concluded that use of saturation monitoring resulted in fewer admissions to the NNU and a reduction in the use of oxygen at resuscitation[41].

The optimum level of oxygen saturation in preterm infants on the neonatal unit is continually being studied. Tin et al compared babies of <28 weeks gestation who were assigned to having saturation targets either between 88-98% or 70-90%. They concluded that maintaining higher saturation targets in this population resulted in more ROP, longer duration of ventilation and more chronic lung disease. There was no difference in survival or neurodevelopmental outcome between groups at one year. This data should encourage clinicians to reduce the amount of oxygen given to these babies[42].

Askie et al performed a study where infants were assigned to either maintaining their saturation levels between 91-94 % or 95-98% for the duration of their reliance on supplemental oxygen therapy. These infants were followed up at 1 year of age and they concluded that maintaining higher oxygen saturation targets did not effect long term neurodevelopmental outcome but had resulted in the infants requiring oxygen for longer and therefore a longer hospital stay [43]. From this it can be concluded that infants can manage with lower saturation targets without it negatively impacting on their long term outcome. It also has the positive effect of not exposing them to excess oxygen for as long, with its inherent risks, along with a better outcome financially through the shorter hospital stay.

A study from the USA has also confirmed these findings. They recruited 502 infants who were divided into two cohorts which were identified following the implementation of a change in clinical practice. This change in practice involved reducing oxygen saturation targets from 92-100% to 85-93%. Lower oxygen saturation targets were shown to reduce the incidence of chronic lung disease and

any stage of ROP (but not the incidence of grade 3 or 4 ROP) and did not impact on neurodevelopmental outcome[44].

Within the UK the BOOST 2 trial has just stopped recruiting infants. This study aimed to compare two different oxygen saturation target levels within infants born at <28 weeks gestation. These target levels were set at 85-89% and 91-95%, although the monitors were offset, to enable alarm limits to be set at the same values regardless of target levels. This enabled the nursing and medical staff caring for the infants to be blinded as to what level of oxygen saturation the infants were actually maintaining. The infants shall be assessed at 2 years of age for the primary outcome of mortality or major neurodisability, along with secondary outcomes of ROP, CLD, growth and general health. These UK results shall be combined with other studies taking place across the world to increase the power of the study and to enable definitive conclusions to be made regarding optimal saturation targets within the NNU. (http://www.npeu.ox.ac.uk accessed on 07.02.2011). Unfortunately the trial was stopped early as there appears to be a significant negative affect on short term mortality in the lower saturation group. The study group have emphasised that this action has been taken on the basis of short term outcomes only and the long term follow up is still planned to take place and this should inform the discussion of oxygen usage further.

One of the difficulties in assessing saturation levels is knowing whether they actually correlate with the infants PaO2. Castillo et al studied 976 paired PaO2 and saturation recordings for 122 neonates. They found that when an infants saturation level was recorded as above 93% 59.9% of infants had a PaO2 of >80mmHg. This level is potentially detrimental to a preterm infant. This quantity of hyperoxia can be reduced to only 4.6% by lowering oxygen saturation target ranges to 85-93%[45].

There is evidence to suggest that the target ranges for oxygen saturation monitoring should vary according to the age of the infant. Chow et al produced a significant reduction (4.5% of infants <1500g in 1997 to 0% in 2001) in the amount of ROP needing treatment by implementing a strict guideline of maximal oxygen saturations of 93% if <32 weeks and 95% if >32 weeks. This policy also included detail about the weaning and increments of oxygen in line with optimal oxygen saturation targets. The aim was to use small increments and decrements in inspired oxygen to minimise periods of hyperoxia and hypoxia[46].

In conclusion there is still much debate regarding oxygen usage in resuscitation and the oxygen saturation target values on the neonatal unit. There appears to be overwhelming acceptance that too much oxygen is bad and so there is a trend to reduce the amount of and increase the stability of oxygen delivered to the infants in the NNU. Exactly what levels are safe has still to be defined and hopefully the results of Boost 2 will inform UK practice.

White Matter Injury

History Of White Matter Injury

In 1861, Little described asphyxia as one of the causes of morbidity in babies delivered prematurely. Virchow first described periventricular leukomalacia (PVL) in 1867 as a form of congenital encephalomyelitis. These babies had pale softened zones of degeneration within the cerebral white matter. Histologically, this consisted of glial hyperplasia and foamy macrophages as well as tissue degeneration and necrosis. There was a correlation between this injury and the presence of maternal infection, as many babies who were born prematurely had mothers infected with syphilis (great pox) or small pox[47].

In the 1870s, Parrott expanded the clinical description to include hyperactivity, spasticity, coma, convulsions and respiratory difficulties. He noticed that haemorrhage and infarction seemed to contribute significantly to these lesions. His theories of causation were nutritional factors and circulatory disturbance, rather than inflammation as proposed by Virchow[47].

In 1932, Rydberg first postulated that the white matter damage might be related to poor cerebral blood flow. He proposed that there was a link between reduced blood pressure and regional changes in vessel diameter[47].

Banker and Larroche first used the term periventricular leukomalacia (PVL) in 1962[48]. In post mortem brains, they noticed that the damage affected certain areas of the white matter: the zone within the subcallosal, superior fronto-occipital and superior longitudinal fasciculi; the external and internal sagittal strata of the temporal and occipital horns of the lateral ventricles and the corona radiata. Damage was evident within a few hours of a documented insult and it was often bilateral without exact symmetry. They put forward three theories regarding the causes of PVL: effects of cardiopulmonary disease, placental abnormalities and a severe anoxic episode prior to delivery. Further studies of anoxic stimuli have confirmed that the damage is evident in white matter only and not grey matter[49].

Classic cystic PVL causes a diplegic type of cerebral palsy, which is well documented[50]. This is due to damage sustained in the internal capsule, which is situated within the periventricular area. It is now evident that many preterm survivors exhibit more subtle cognitive and behavioural deficits, which are likely to be related to a more diffuse type of white matter damage [51,52].

The most important way to prevent adverse neurological outcome in preterm infants is to increase gestational age at delivery and reduce the incidence of intra-ventricular haemorrhage and cystic PVL[53]. These two pathologies are the most significant contributors to delayed motor development in the preterm neonate. The discussion regarding haemorrhage is outside the scope of this chapter; thus the following discussion shall focus on PVL and white matter injury.

Peri-Ventricular Leukomalacia

The preterm brain is exquisitely sensitive to damage for a variety of reasons. Firstly, it has a relatively low cerebral blood flow (1.6ml/100g/min when compared to adult CBF of 50ml/100g/min). This means that only small alterations in systemic blood pressure can lead to an alteration of CBF and ensuing ischaemic or reperfusion damage. The vascular anatomy is also very basic with plenty of under-vascularised border zones due to lack of vessel branches and anastomoses. Secondly, the preterm brain may be subjected to inflammation in the form of perinatal infection. These two factors can lead to excitotoxicity and free radical damage. The main cell in the white matter of infants born extremely preterm is the pre-oligodendrocyte. This is especially sensitive to attack due to its ineffective antioxidant systems and the accumulation of free iron within the cell[54].

PVL can be described as being either cystic or non-cystic. Cystic PVL is less common than previously due to improvements in perinatal care resulting in fewer infants being born following significant intrauterine hypoxia and their neonatal courses being more stable than previously through the use of antenatal steroids and surfactant. The damage originates as areas of focal necrosis that then evolve in to cysts. The cysts occur in deep white matter with a loss of all cell types[54].

Cystic PVL consists of well-described foci of damage 2-6mm diameter within 15mm of the ventricle wall. Most commonly these are anterior to the frontal horn, lateral corners of the lateral ventricles, and lateral regions of the trigone and occipital horn. PVL begins with necrosis within 3-8 hours of the insult resulting in nuclear pyknosis, tissue vacuolation, increased eosinophilia and swollen axons (spheroids) along with necrosis of all cellular elements. Within 12 hours astrocytic proliferation, capillary hyperplasia, microglial proliferation and lipid laden cells accumulate. Cavitation occurs within a few weeks. The healed lesions stain faintly for MBP as myelin producing oligodendrocytes are not present within the lesions[55].

Cysts may be noticed on ultrasound from day one of life suggesting an insult up to 2 weeks prior to birth. Taking a few weeks to evolve, these cysts may also be seen at any time during the postnatal period reflecting the hypoxia–ischaemia events occurring during the transition following birth. These cysts generally affect the motor tracts resulting in the classic diplegia seen in these infants[56].

Cystic PVL is much less common now than previously due to improved obstetric care and survivors of preterm birth having a generally more stable postnatal course than before due to the use of antenatal steroids and surfactants. As a result classical cystic PVL is rarely seen. But a second form of white matter injury is increasing its prevalence. This is described as non-cystic white matter injury. With modern imaging techniques this injury is becoming more commonly identified. Inder et al demonstrated that in a series of 96 infants, who had an MRI performed at term equivalent age, only 4 had cystic injury whereas 34 had non-cystic white matter damage[57]. This pathology is now being extensively investigated and is likely to contribute significantly to the significant neurodevelopmental morbidities seen in the group of premature survivors.

Non-cystic white matter injury is characterised by pyknotic glial nuclei and gliosis. This results in impaired myelination. Its causes are multiple but include hypoxia reperfusion and cytotoxic cytokines released during infection or ischemia[54]. The greatest time of damage is between 24-32 weeks, which is the period of most active myelination synthesis. This implies that oligodendrocyte precursors have some role to play here[58].

This diffuse white matter damage is characterised by inadequate myelination due to disruption to normal oligodendrocyte processes. Oligodendrocytes are a very vulnerable cell line due to their high metabolic demands. The late oligodendrocyte precursor cells are at particular risk of damage[58]. This cell death is thought to be partially free radical mediated as it has been shown that the cells have a reduced level of the antioxidant glutathione. Oligodendrocyte development is discussed further in the chapter on human pathology.

Non-cystic PVL may be linked to the development of the vascular supply of the cerebrum leading to watershed areas, which are under-supplied by the vasculature. The preterm infant is less able to control its cerebral blood flow so the brain is at risk of hypoperfusion in any situation resulting in a drop in systemic blood pressure[59]. This produces hypoxia and ischaemia resulting in white matter damage.

This alteration in cerebral blood flow has been studied extensively in sheep models of prematurity. In one such study the sheep's blood pressure was reduced by 25% and then restored with a bolus of fluid. This group showed that even with restoration of systemic blood pressure the cerebral blood flow in the white matter was still significantly reduced even though blood pressure had been restored[60]. This implies that there are other factors that predispose the white matter to injury at times of poor perfusion.

Radial glial cells (astrocytes) are the first glial cells to be identified. They are stained using glial fibrillary acidic protein. They are believed to help organize the cerebral cortex by providing guidance to migrating neurons. They are transient and disappear at the end of neuronal migration and become fibrillary or protoplasmic astrocytes. From 15 weeks they are involved in signalling to guide the development of the vasculature. With astrocytes having such an important role it is easy to see how if they become damaged in the development of PVL, the overall development of the brain can be significantly compromised[54].

It has been shown that in areas of diffuse white matter injury there is significant damage to the axons resulting in loss of white matter and as a result loss of trophic factors for the ongoing development and growth of neurons. The apoptotic marker fractin was found in areas of the brain distinct from focal PVL lesions. These areas showed reactive astrocytes and activated microglia but not actual necrosis. This shows that cell lines are affected outside focal points of injury thus contributing to a more diffuse type of injury and probably is implicated in much of the neurodevelopmental morbidity seen in survivors of preterm birth. This diffuse axonal damage may be related directly to hypoxia ischaemia or secondary to the death of neuronal cell bodies in the cortex whose axons traverse the white matter[61].

It is known that in PVL there is a reduction in oligodendrocyte number and reduction in myelin. What is not known is whether this is related to arrested maturation of the oligodendrocyte (OL), damage to the myelinated fibres or altered axonal – OL signalling. Billiards et al performed a study on 18 PVL brains and 18 matched controls to try and establish at what stage of the myelination process the damage was occurring. They showed that the number of OLs was the same regardless of injury but there were differences between the amounts of MBP expression. This indicates loss of some OL processes, persistence of less mature OLs and abnormal MBP expression around periventricular necrotic foci. They concluded that the lack of myelin in brains of survivors with PVL was related to four factors. Firstly inadequate repair following injury due to lack of movement / increased numbers of pre OLs. Secondly, the failure of maturation of OL progenitors into the mature phenotype. Thirdly, an inability of the mature OL to produce enough myelin after this injury. Finally, a primary axonal failure with resultant defective axonal signalling for myelin production. The MBP expression in these brains was also abnormal with it being found in the perikaryon not the cell processes. This suggests some form of defective signalling within the tissue as well leading to abnormal myelination[62].

Squier and Keeling first described in detail the incidence of prenatal brain injury in a group of stillborn or early neonatal death brains in 1991. They showed that 44% of the cohort of stillbirths had evidence of injury that was probably circulatory in origin. 26% had widespread ischemic white matter changes including reactive astrocytosis, macrophage infiltration, karyhorrhexis and endothelial duplication or swelling. They concluded that 36% of those infants who died within the first week of life had white matter damage that was significantly prenatal in origin and not related to factors present around the time of delivery. They also surmised that this damage may lead to altered brain development specifically affecting the processes of myelination [63].

Some of the abnormality in brain architecture and diffuse white mater injury is related to damage to the subplate neurons. These cells form synaptic contact for waiting cells before the development of the cortical plate and guide axons in to the correct position during cortical organisation and synaptic development. As a result any damage to these subplate neurons, as happens in white matter disease, has a significant affect on the general development of the brain[64]. Damage to the subplate neurons leads to poor visual development as these connections do not develop in the correct way. The fact the preterm infants with white matter injury often have difficulties especially with cortical visual impairment and visuo-spatial tasks confirms this[65].

Clinical Implications

Survival following preterm birth has greatly improved over the past two decades. However, in spite of this improved survival, the rate of cerebral palsy in these infants remains constant [66]. Recent data from Europe suggests a possible reduction in incidence but only in the birth weight group of 1000-1499g[50]. Around 10% of survivors with a birth weight of <1500g exhibit some signs of cerebral palsy[67]. These are generally associated with gross changes on cranial ultrasound of significant intra-ventricular haemorrhage, post haemorrhagic hydrocephalus or cystic peri-ventricular

leukomalacia[68]. Cerebral palsy in premature infants is often in the form of a diplegia related to damage to the internal capsule following periventricular white matter damage[50].

The initial Epicure study took place in the UK and Ireland in 1995 and collated data about all births from 20 to 25 weeks. They recorded 4004 births and 843 infants were admitted to a neonatal unit. This group initially assessed perinatal factors that predicted neonatal outcomes including death. They concluded that factors that contributed significantly to having an abnormal cranial ultrasound scan at discharge were lack of antenatal steroids and transfer within 24 hours of birth. Only 314 were discharged home and six children then died before any long term follow up was completed. As a result the studies of long term outcome from this cohort are based on 308 infants [69].

Only 50% of the survivors born between 20 and 25 completed weeks were completely free of disability including cognitive and behavioural problems when seen at 2 years of age. 23% of this group were classified as having severe disability on the Bayley scale testing for psychomotor and cognitive development[67].

To assess the full extent of disability following survival from preterm birth, it is necessary to follow the children for many years. Further follow up of these children from the Epicure study at the age of six has now been done. An assessment of their rates of disability revealed that 34 % had mild disability. This was defined as having some mild neurological signs, which did not impact on functioning, squints or refractive errors. 24% had moderate disability, defined as the expectation that reasonable independence would be reached, ambulant cerebral palsy, IQ 2-3 SD below the mean and correctable hearing or visual loss. 22% had severe disability defined as being highly dependent on carers and non-ambulant cerebral palsy. Disabling cerebral palsy was present in 12% of this cohort[70] This group have gone on to show that even in infants without cerebral palsy these survivors perform significantly worse than their peers in a range of tasks assessing executive functions. These tests include posting coins, heel standing, copying designs and finger tapping. They conclude that these subtle deviations from normal significantly impact on the educational outcomes of these children[71].

The Epicure cohort has since been assessed at 11 years of age. At this stage 60% of the cohort required additional support in school with a third having a statement of educational need. The children were particularly weak in mathematic skills compared to reading. The investigators also raised concerns about how many children born preterm end up the academic year ahead of where they would have been had they been born at term. They hypothesise that this may explain a small amount of discrepancy seen in attainment between them and their term born peers, but acknowledge that most of the problems have a more organic cause[72].

Hille et al assessed 408 ELBW children at school age from Europe and North America using a 118 point behaviour checklist. This assessed indictors for ADHD as well as social skills and anxiety levels. There were no differences in scores between countries of origin but there were significantly poorer outcomes in the ELBW cohort

than the control population born at term [73]. Bhutta performed a meta-analysis of 16 studies looking at behavioural outcome in preterm infants at school age and found a relative risk of 2.64 (95% CI 1.85-3.78) of developing ADHD[74]. Botting et al. who have followed up 137 VLBW survivors also demonstrated this increased risk in 23% of children compared to 6% in the control group. They also demonstrated an increase in the prevalence of depression in these children: 28% compared to 9% in the control group[75].

The ex-preterm population of infants often need extra assistance when at school. Hille et al evaluated 813 children who had been born at <32 weeks or <1500g. They found 19% needed special schooling. 38% of those in mainstream education required some type of additional support to keep them there and 32% were at least a grade below their peer group[73]. This group of children had a lower IQ score at 12 years than their peers. This included problems with poor vocabulary, comprehension and mathematic skills [75].

Powls et al examined forty-seven VLBW children attending mainstream school at the age of twelve. 34% of these children had a significant motor impairment when examined using the motor assessment battery for children, which mainly affected tasks of manual dexterity. There was little difference between the two groups when the results were compared to the perinatal history. Perinatal factors examined included cranial ultrasound reports, seizures, and episodes of sepsis or days of ventilation. This implies more subtle neurological damage is occurring in these babies that is not predicted from the neonatal course[76] and these educational difficulties continue into adulthood[77].

Clinical Factors Contributing To White Matter Injury

There are many clinical factors that contribute to white matter injury. These include:

Antenatal:	genetic factors, intra-uterine growth retardation, multiple pregnancy, infection.
Perinatal:	hypoxia–ischemia.
Postnatal:	cardiovascular instability, infection, respiratory distress syndrome and hyperoxia,

Some of these factors will be discussed in detail below.

Intrauterine Growth Retardation / Poor Postnatal Growth

Adequate fetal growth is essential for normal development. Inadequate function of the placenta not only leads to chronic intrauterine hypoxia but also altered transport of nutrients to the fetus which then affects both somatic and brain growth. A specific profile of difficulties in coordination, executive functioning, language abilities and creativity has been described in children with intrauterine growth retardation (IUGR) by a group from Israel. These children were deemed to have experienced third trimester growth restriction as shown by a discrepancy in expected cephalisation index (head circumference / birth weight $x10^2$) meaning they had asymmetric growth retardation with brain sparing. The placentas of 85% of this cohort demonstrated some form of vascular pathology. This population demonstrated significantly lower

neurodevelopmental scores, IQ and overall school achievement than aged-matched control subjects. Children with overt cerebral palsy were excluded from the study[78].

With the improvement in magnetic resonance imaging techniques much work has been done to assess the actual structural problems in the brains of these IUGR infants. Tolsa et al showed that IUGR infants have less grey matter than appropriately sized infants matched for gestational age. These infants were also found to have problems with attention and interaction[79]. The same group specifically assessed the size of the hippocampus and found this to be smaller in infants with IUGR which correlates with the clinical findings of the infants having issues with attention.[80].

Evidence of placental lesions in IUGR infants who also have white matter damage on cranial ultrasound lends weight to the argument that the cause of the abnormal brain development is vascular in origin[81].

There is evidence of brain injury being prenatal in origin and related to placental insufficiency and growth restriction. In studies of fetal sheep the brain weight was noted to be smaller than appropriately grown fetuses and although often there was no overt white matter damage there was reduction in myelination in the growth restricted brains[82].

Postnatal growth rate may also be extremely important. Inappropriately sized VLBW infants are in negative energy balance in the early days of their postnatal life and often take weeks to gain weight at the normal intra-uterine rate. As a result they may be undernourished at a crucial stage of brain development which may have a significant impact on their neurodevelopmental outcome[83]. Infants who demonstrate adequate catch up growth in the first decade of life have a lesser incidence of neurodevelopmental problems later in life when compared to those infants without adequate catch up growth[78].

Multiple Pregnancy

Multiple pregnancies have a higher risk of cerebral palsy, with twins making up 10% of the cerebral palsy population, but only constituting 1% of live births[66]. A meta-analysis on the subject identified a significant increase in neurological morbidity for both live-born twins and those survivors following the demise of the other twin. There is a marked difference between the outcomes of mono-chorionic and di-chorionic twins. Following the demise of one twin in a mono-chorionic pregnancy 18% of survivors show some form of neurological deficit. In di-chorionic pregnancy this risk is 1%. The mechanisms relating to this are not completely clear but one probable factor is that there is an excess of inflammatory cytokines following fetal demise which may be transferred to the surviving twin. In mono-chorionic twins there is a belief that after the demise of one twin the blood preferentially flows through this low resistance circuit leading to hypotension and ischaemia in the surviving twin. This probably explains some of neurological deficits seen in these survivors. Surviving pairs of twins from a mono-chorionic pregnancy have an odds ratio of developing some neurological abnormality of 4.07 (95% CI 1.32-12.31)[84].

Chorioamnionitis

Intrauterine infection associated with chorioamnionitis is associated with an increased risk of cerebral palsy in babies born at term[85]. The link is less clear in premature infants who have many other confounding factors[66]. Wilson Costello examined 72 infants with cerebral palsy and 72 without from a cohort of VLBW babies. Chorioamnionitis doubled the risk of any form of neurological impairment, as did neonatal septicaemia. In the group with just cerebral palsy without other impairments this factor was deemed less significant. This could be interpreted to show that the infection causes a generalised insult which leads to a range of subtle impairments along with the cerebral palsy but has no impact specifically on the rate of cerebral palsy within this group of VLBW infants [86].

This mechanism of injury is related to an up-regulation of the inflammatory cascades. Bacterial infection results in a release of endotoxins (lipopolysaccharide) which can bind to specific cell membrane receptors to activate microglia[87]. Microglia, activated by lipopolysaccharide, have been shown to release reactive oxygen and nitrogen species contributing to cell death[88]. This link has been proved in animal studies with the use of endotoxin and lipopolysaccharide to induce inflammation. The animals infected demonstrated white matter injury at post mortem[59]. If the brain is exposed to an ischaemic insult on top of this then the amount of damage is increased[89].

There is plenty of clinical evidence to implicate infection / inflammation with cystic PVL. A systematic review by Wu showed that chorioamnionitis resulted in a RR of cerebral palsy of 1.9 (95% CI 1.5-2.5) and a RR of cystic PVL of 2.6 (95% CI 1.7-3.9). As is to be expected the data was pooled from a heterogeneous group of studies assessing infants with different diagnostic criteria for chorioamnionitis (clinical or pathological), different gestations of infants and different diagnostic criteria for cerebral palsy[90]. In spite of this heterogeneity, as is typical of most studies involving infection, this evidence is compelling and has large implications for clinical practice.

It has been demonstrated that preterm infants with white matter injury have higher CSF levels of several inflammatory cytokines (IL6, IL-10 and TNF-α) when compared to a similar group without white matter injury. This implies a role of infection / inflammation in the development of white matter injury[91]. How these cytokines cause damage is less clear. It is thought they may have a role in disturbing vascular endothelium resulting in injury through vascular endothelial growth factor[92,59]. In neuropathology specimens of PVL there is evidence of TNFα and IFNγ present in microglia and macrophages. IFNγ has been found in astrocytes in diffuse PVL. The IFNγ receptor is found on pre-Ols and binding of IFNγ can lead to cell death[54].

There is evidence that an elevation of some of these cytokines is in fact protective. IL6 has been demonstrated to inhibit neuronal death in instances of middle cerebral artery occlusion[93].

Ischaemia

The preterm brain is especially susceptible to ischaemia because of poor development of its vascular supply and impaired regulation of cerebral blood flow. The preterm brain is perfused by ventriculofugal and ventriculopetal arteries. These long penetrating vessels have few in the way of anastomoses or branches and as a result there are large areas of the deep white matter that are relatively under perfused. The vessels in this area develop gradually over the last 16 weeks of gestation so the extremely preterm infant is very vulnerable to ischaemic injury[94].

To compound this anatomical inadequacy the preterm brain has a very low cerebral blood flow and is pressure passive. As a result a small reduction in systemic blood pressure can result in a failure of blood flow to certain areas of the brain. The cerebral blood flow of a preterm infant can be as low as 1.6ml/100g/min, compared to an adult's that is 50ml/100g/min. This lack of control of cerebral blood flow is related to immaturity of vasoregulatory mechanisms but also can be affected by the general clinical status of the infant. It is recognised that septic neonates have less ability to maintain their cerebral circulation than well neonates[54].

Cerebral blood flow can also be affected by hypocarbia as this results in vasoconstriction. Shankaran studied 905 infants weighing less than 1250g at birth. This group assessed the cumulative index of hypocarbia and related this to presence of PVL. They monitored arterial blood gases every 6 hours and calculated the cumulative hypocarbia by (35mmHg –PaCO2) x hours. Hypocarbia within the first week of life was associated with an odds ratio of developing PVL of 5.6[95].

Birth Asphyxia / Hypoxia-Ischemia

Birth asphyxia remains the leading cause of neonatal death worldwide. This condition affects 1.8 to 47 per thousand live births. The picture of asphyxia is complicated by the knowledge that many infants have evidence of white matter injury that significantly predates the onset of labour[96].

In the mature fetus, brain damage is induced by severe intrauterine asphyxia due to a reduction in blood supply from the uterine or umbilical circulation e.g. in the situation of cord prolapse / uterine rupture. Initially the fetus compensates for this change in environment by redistributing blood flow to the vital organs: brain, heart and adrenals. However, this compensatory mechanism may eventually fail with a prolonged insult. This usually affects the parasagittal region of the cerebral cortex and the basal ganglia. This leads to a reduction in the metabolic processes within the brain and an influx of intracellular calcium, which results in the cell damage. This process is increased by an elevation in the amount of the excitatory neurotransmitter glutamate present, resulting in more cell damage.

This is followed by a second stage of damage caused by a period of hyperperfusion due to hypercapnia and hypoxia. The tissue acidosis leads to vasodilation related to the hypercapnia. Recurrent short bursts of ischaemia do not result in hypercapnia and consequently, do not show this vasodilation but do lead to a prolonged reduction in blood flow.

The vasodilation and increased tissue perfusion leads to a release of oxygen free radicals. This in turn leads to the synthesis of nitric oxide. Usually 80% of these free radicals are successfully reduced by the mitochondria and do not cause any lasting damage. But in this period of reperfusion there is an increase in free radicals resulting in saturation of the anti-oxidant defences. This excess of free radicals results in oxidative damage and consequently apoptosis. Therapies to reduce injury are aimed at reducing the amount of apoptosis that is occurring. This has been done experimentally by the administration of caspase inhibitors which have lead to a reduction in neurological damage[97].

Following the initial insult there is a further stage of damage that occurs several hours later. This happens after a period of presumed stability. This period is related to energy failure within the brain due to a lack of glucose and ATP. The various trials of brain cooling are aimed at affecting this second phase of damage. It is believed that by cooling the brain this reduces metabolic demand and thus limits damage. Hypothermia appears to reduce the free radical response to injury as well as reducing excitatory neurotransmitters[98].

The largest trial of total body cooling has now been published in the New England Journal of Medicine. This study set out to establish if cooling infants to a temperature of 33.5°C for 72 hours when they had experienced perinatal asphyxial encephalopathy had any effect on neurodevelopmental outcome. The multinational group recruited 325 infants, of these 163 were cooled. They showed that cooling had no effect on mortality or severe disability rates within the cohort but there was an improvement in neurodevelopmental outcomes of the survivors from moderate asphyxial injury. This has lead to cooling becoming part of the expected standard of care for these infants[99].

There is evidence from fetal sheep that multiple short bursts of ischaemia are more damaging that one prolonged period. Sheep were exposed to either 3 occasions of 10 minutes injury either 1 hour or 5 hours apart or to 30 minutes continuous ischaemia. Those with the frequent short bursts with less time for recovery had greater neuronal loss and their EEG took longer to return to normal. The authors concluded that this showed that short bursts sensitise the brain and make it less tolerant of further exposures to injury[100].

Hyperoxia

In 1980, Ahdab-Barmada published a paper linking hyperoxia with pontosubicular necrosis (PSN). PSN is damage to the pontine base and subiculum of the hippocampus, associated with karyorrhexis in neurons and astrocytes[101]. They reviewed 64 neonatal autopsies where there had been multiple PO_2 determinants during life. They divided the group into those with a PO_2 level of <150Torr and those with a level >150Torr. PSN was present in 36/37 in the higher oxygen tensions and none in the lower oxygen tensions[102].

Collins etc el looked at a variety of ventilatory parameters in relation to cerebral palsy at 2 years of age. They identified 657 infants who had blood gases taken during their stay on a NNU between 1984 and 1987. Hypocapnia was defined as <35mmHg

and hyperoxia was defined as >60mmHg. They concluded that having either hypocapnia or hyperoxia increased the risk of disabling cerebral palsy 2-3 fold[103].

These results have been replicated in a study from Canada. This group demonstrated that hyperoxia alone following asphyxia increased the chances of an adverse outcome (death, severe cerebral palsy or cerebral palsy with sensory impairment) OR 3.85 CI 1.67-8.88. In conjunction with hypocapnia this increased to OR 4.56 CI 1.4-14.9[104,105].

Hyperoxia is known to cause apoptosis in the brains of rat pups. This damage is related to the age of the pups with younger pups demonstrating more damage than older pups. This is due to the hyperoxia affecting the preoligodendrocytes more than the mature oligodendrocytes. This is the process that is seen in the preterm brain [106]. Physiological hyperoxia has been shown to affect myelination and reactive astrocytosis in a rodent model of preterm brain injury[107].

Ahdab-Barmada showed that newborn rats exposed to only three hours of 100% oxygen demonstrate neuropathological changes akin to those seen in asphyxiated neonates, namely PSN. Physically these rats behaved differently. They were hyperactive, hyperthermic and were smaller than age related controls; some had periods of apnoea and bradycardia. The type of histological damage was very different in those pups exposed to hyperoxia when compared to the damage seen in the animals exposed to hypoxia in the same experiment. The hyperoxia group showed significantly more evidence of a spongy neuropil and neuronal karyorrhexis, which may be free radical mediated. They also noted an increase in the presence of free fatty acids which could be due to lipid peroxidation possibly from free radical processes[101].

Usually the respiratory chain utilises oxygen and antioxidants absorb the excess to limit the amount of free radicals available to damage local tissues. At the time of either a reperfusion event or hyperoxia there is an excess of oxygen available to the mitochondria to reduce and use. This results in an excess of superoxide radicals that can then lead to free radical injury on their own or they may react with nitric oxide to become the highly toxic substance, peroxynitrite[108]. Manganese superoxide dismutase, an antioxidant, is found in much higher concentrations within mature oligodendrocytes than immature ones. This partly explains the mature OLs resistance to damage[109].

Evidence of free radical involvement in PVL has been demonstrated by the presence of oxidative (hydroxynonenal) and nitrosative (nitrotyrosine) attack using immunohistochemistry. Evidence of both mechanisms has been found in pre-OLs and astrocytes[110]. This vulnerability to free radical attack is partly due to the immature development of antioxidant defences and the presence of free iron within the CNS. The iron is essential for pre-OL maturation and is present in greater quantities following any form of intraventricular haemorrhage. This combines with superoxide to form the hydroxyl radical. There is also an increased amount of iNOS present in the microglia that are present in diffuse PVL, in particular in the astrocytes[54].

As has been shown a range of factors can affect the type and severity of brain injury experienced by survivors of premature birth. This body of work is specifically interested in the role of fluctuating oxygen tensions in the development of brain injury.

Imaging Techniques

An increasing number of MRI studies on preterm survivors are improving the understanding of white matter injury in vivo. Diffuse white matter damage is often present on an MRI examination that is not easily recognisable on a cranial ultrasound [111].

Cranial ultrasound is cheap and easy to perform compared to an MRI and, as such, it is more widely used in clinical practice. Some abnormalities within the white matter are picked up by ultrasound. Initially there is evidence of congestion (flare) particularly in the anterior and posterior periventricular areas. This flare may resolve completely or develop into cysts [112].

MRI demonstrates cystic lesions, enlarged ventricles, delayed myelination, diffuse excessive high signal intensity (DEHSI) areas within the white matter and cortical atrophy in preterm survivors[113]. Inder et. al. studied 96 preterm survivors and found 34 to have non-cystic white matter abnormalities on MRI, whilst only 4 had evidence of PVL on cranial ultrasound[57]. This confirmed the findings of Maalouf's study, that ultrasound was useful at detecting haemorrhages and infarctions with a predictive probability (PP) of 0.8 (0.7-0.9) and 0.85 (0.76-0.94) respectively. The presence of severe white matter injury was very well predicted with a PP of 0.96 (0.92-1). Prediction of the presence of DEHSI in white matter was less accurate with a PP of 0.54 (0.42-0.66)[114]. As a result parents may be falsely reassured by a normal cranial ultrasound whereas an MRI at term may demonstrate DEHSI and therefore more accurately inform prognosis for these infants.

Mirmiran et al compared cranial ultrasound in the first two weeks of life with MRI at term for predicting cerebral palsy at 20 and 31 months. They had a cohort of 61 children who had had early cranial ultrasound and then MRI at term. They showed that cranial ultrasound had a sensitivity of 29% and specificity of 86% of predicting CP at 20 months of age as compared to the MRI, which had a 71% sensitivity and 91% specificity[115]. For teams who do not have such easy access to MRI facilities this may be disappointing as a normal ultrasound may be falsely reassuring. However, at least there is comfort in knowing that if the ultrasound images are abnormal there is a high chance that there will be problems in the future. This reflects the fact that ultrasound is acknowledged to be good at identifying haemorrhages or large areas of damage but less good at showing a more diffuse injury.

A more diffuse non-cystic injury is recognised by "flare" or brightness on cranial ultrasound, which may resolve or persist. Its persistence may be related to the ongoing cognitive problems seen in survivors of preterm birth. This is because the

damage leads to abnormal neuronal migration and organisation. There is good MRI evidence to back up this theory. Diffuse Excessive High Signal Intensity (DEHSI) is seen on the MRI images of around 75% of survivors of preterm birth. It manifests itself as increased signal intensity on T2 weighted images within the white mater of the brain. It has been confirmed that this marks areas of abnormal white matter. Diffusion weighted imaging has shown that in these areas there is altered water diffusion coefficients within the fibre tracts implying some alteration in their development[114]. The rates of DEHSI are similar to the rates of cognitive impairment seen in the survivors of preterm birth. Dyet et. al. performed 327 scans on 119 infants born between 23 and 30 weeks of gestation. At term equivalent 80% of the infants had DEHSI, which was the most common abnormality present on MR imaging of this cohort. Ventricular dilatation was the next most common abnormality affecting only 30% of the cohort. The presence of severe DEHSI vs. no DEHSI without other abnormality represented a reduction in DQ of 14 points (p=0.023) on follow up. In the discussion of their results they alluded to the controversy regarding the significance of DEHSI on an MRI scan. There is a lack of consensus as to whether DEHSI represents delayed myelination, which may be a marker for future damage, or actual abnormal myelination[51].

Inder et al performed MRI scans on infants born at term and preterm infants at term equivalent age to assess cerebral tissue volumes. They showed significant reductions in cerebral tissue volumes in all areas (cortical grey matter, deep nuclear grey matter and myelinated white matter) except unmyelinated white matter. This provides evidence that damage to the white matter is inextricably linked to damage to the grey matter due to the role of white matter in supporting growth and development of the brain[116].

There is evidence that in children, who had been born prematurely there is disorganised white matter present when they are scanned at 12 years of age. These children had been selected as having no white matter injury, as defined by cranial ultrasound, during the neonatal period. The 29 children assessed had birth weights of between 600 and 1250g. At 12 years of age there were significant differences in verbal and performance IQ and a developmental test of visual motor integration between those infants born preterm and matched term control subjects. On diffusion tensor imaging they had significantly reduced fractional anisotropy (a method of measuring fibre tract organisation by assessing the movement of water molecules within the tract) and reduced white matter volumes[117]. This helps explain why many children born prematurely who have normal neonatal cranial ultrasound images do have some degree of learning difficulty in later life. This may be due to the lack of sensitivity of cranial ultrasound in diagnosing subtle white matter injury, acknowledging that few infants are imaged using MRI technology. Alternatively it could be a genuine finding that simply being born prematurely appears to disrupt the normal development of the brain. Many factors could be involved in these processes and the great challenge is to ascertain which factors are the most important and which ones we as clinicians can alter to improve outcome for these infants.

It may not just be postnatal factors leading to these abnormalities. Bax et al reported on the MRI findings of a group of children with cerebral palsy. Many of these

children had no single identified risk factor for brain damage but it was concluded that there could have been a group of less significant factors having a synergistic effect leading to the white matter damage seen. Much of this is deemed to have occurred early in gestation so unfortunately is probably unmodifiable [118]. This conclusion is backed up by the work of Becher et al who described a series of neuropathology from neonatal deaths where many of the brains showed evidence of damage that definitely predated the onset of labour. They suggested that with some antenatal damage the infants are set up to tolerate labour less well leading to poorer outcomes[96].

Correlating MRI findings with neurodevelopmental outcome is important. Woodward et al showed that an increased amount of white matter damage evident on MRI scanning is related to a worse neurodevelopmental outcome. In their study of 167 preterm infants scanned at term equivalent, they demonstrated the following odds ratios associated with moderate to severe white matter MRI abnormalities: cognitive delay 3.6, motor delay 10.3, cerebral palsy 9.6 and neurosensory impairment 4.2. Within this cohort were 6 infants who had severe MRI abnormalities of which 2 of these infants were completely normal at a two-year follow up. Similarly, in the normal MRI group of 47, 2 had severe motor delay and 2 had a significant neurosensory impairment. The total in this group for any neurological abnormality was 15%, two thirds of which were related to familial causes of neurodevelopmental delay[119].

With the increasing use of both fetal and postnatal MRI along with the increased improvement in image quality that comes with the newer 3T scanners hopefully some of these questions shall be answered and MRI will become an even better predictive marker for outcome than it is already.

Cerebral Vasculature

The Process Of Angiogenesis

Vasculogenesis is the process that forms the basic vascular plexus from which further vessels sprout. The vascular plexus is mesoderm in origin. Angioblasts develop within blood islands from haemangioblasts and these go on to create the basic vessels.

Angiogenesis is the process by which there is sprouting from these basic vasculogenic vessels to build the more complex vascular architecture needed for life. The majority of cerebral blood vessels are formed through the process of angiogenesis[120].

The vascular branching is accompanied by the vessels maturation in the form of pericyte recruitment and also an increase in the astrocytic covering of the vessels. Capillaries have a thin basement membrane and are just one endothelial cell thick. The endothelial cells can undergo rapid proliferation or remain static for several years. This is essential in the processes of growth and repair.

Astrocytes are believed to help develop and maintain blood brain barrier properties, especially permeability. Initially the vessels are closely related to the presence of radial glial cells, which are necessary for guiding vessel development. In a rodent model at later gestations, 15th postnatal day, the vessels are well covered in astrocytes. These rodent vessels at all stages of postnatal development are impervious to Evans blue implying the functional maturity of the blood brain barrier from birth[121]. Within the human brain the germinal matrix vessels have reduced astrocyte coverage when compared to vessels of the white and grey matter until 34 weeks gestation. This is one of the reasons why this area is especially fragile in the premature infant and prone to bleeding[122].

Once the vascular network is established this process slows down dramatically. Following this, fresh vascular growth only occurs at times of tissue growth when the vessels need to be elongated to perfuse the additional tissue mass[120].

Vascular Endothelial Growth Factor

Cerebral angiogenesis is under the direct control of Vascular Endothelial Growth Factor (VEGF). This is a soluble protein which is derived from the neuroectoderm and is secreted by neurons and astrocytes[123]. It stimulates growth of endothelial cells and is also integral to their survival. It is present in large quantities shortly after birth and subsequently its presence depends on brain expansion and the need for vessel development. Arai et al established the presence and timing of VEGF expression in a cohort of 16 infants. VEGF was present from 9 weeks of gestation in the germinal matrix and from 17 weeks in the white matter. The white matter VEGF peaked between 24 and 28 weeks[124]. They also found that VEGF was present in the astrocytes surrounding the areas of PVL. VEGF was not present in these cells in normal brain tissue[124].

VEGF is up-regulated at times of hypoxia[120,125,126]. This was demonstrated by a three fold increase in VEGF RNA in rat brains exposed to 8% oxygen for four weeks. It is assumed this is to enable improved oxygenation to hypoxic tissue. The up-regulation of VEGF at times of hypoxia is mediated through the presence of Hypoxia inducible factor (HIF 1α) present in neural cells[127]. This binds to the hypoxia response element within the VEGF gene[128]. HIF 1 α is an important protein, which is secreted in greater amounts at times of hypoxia enabling the shift from aerobic to anaerobic metabolism by induction of glycolytic enzymes. It is very sensitive to hypoxia and as such it is degraded within 5 minutes of re-oxygenation. It has an essential role in regulating angiogenesis demonstrated by HIF 1α null mice that die in utero. A reduction of HIF 1 α results in a reduction in vessel and therefore organ development[129].

VEGF is also up-regulated from the endothelial cells and astrocytes following ischaemic injury [124]. Neovascularisation is seen around areas of PVL one week after injury. In post mortem studies of stroke victims there is often an increase in vessel density around the site of the infarct. This is particularly evident in people who have survived a longer time after the event. The additional vessels may reduce the risk of further infarct[128]. In a rodent model, administration of VEGF 24 hours after the insult resulted in a 35% reduction in infarct size[130]. The disadvantage of the increase in

VEGF is that it is also a permeability factor and causes leakage of proteins through the blood brain barrier[126]. This mechanism is thought to be the cause of the cerebral oedema found in altitude sickness[125,128].

Hypoxia And Its Role In Angiogenesis

Oxygen supply to the brain is maintained by favourable blood flow to different areas as required. Compensatory mechanisms involving hyperventilation and vasodilation result in adequate oxygenation to essential areas during periods of minimal hypoxia.

Prolonged hypoxia leads to an increase in haemoglobin concentration with resultant increased oxygen carrying capacity of the blood. Oxygen availability is directly related to diffusional pressure, a complex interplay between concentration gradient and distance of travel from capillary to cell. This lack of oxygen is compensated for by an increase in the number of capillaries within the brain, which should result in a reduced diffusional distance and lead to maintenance of oxygenation[131,126].

The increase in vascularity is related to both endothelial cell hyperplasia (making more of them, which occurs after a prolonged period of hypoxia) and also hypertrophy (making the current cells larger, which occurs at the initial hypoxia)[131].

Animals reared in a hypoxic atmosphere also demonstrate an increase in PECAM-1 (platelet endothelial cell adhesion molecule) expression. This is an endothelial cell marker and therefore a marker of angiogenesis[132]. This is associated with an increased microvessel lumen diameter as well as an increased number and density of capillaries within the brain[133]. There is altered expression of VEGF in these brains. In normoxia VEGF is found initially in the cortical neurons and then later in the glial cells that envelope the blood vessels. In prolonged hypoxia the neuronal signal continues along with the glial cell expression leading to disruption of the normal development of the vasculature. Exposing animals to 9.5% oxygen resulted in a 40-51% increase in vessel diameter by 24 days. There was also a 30% increase in vessel number at the same time[133].

Hyperoxia And Its Role In Angiogenesis

Treatment of rat mammary tumours with normobaric and hyperbaric hyperoxia results in a retardation of tumour growth. This is partially related to a reduction in vascular density within the tumour and an increase in apoptosis of tumour cells. The diameters of the remaining vessels were increased in response to the hyperoxia. This may be related to the presence of free radicals leading to the apoptosis and inhibition of angiogenesis. There is potentially a role for the down-regulation of VEGF in the presence of hyperoxia. The increased vessel diameter could be indicative of the hypoxic tissue, resulting from lack of blood vessel growth, trying to develop in other ways to maintain tissue oxygenation[134].

Anatomy Of Human Cerebral Vasculature

The medullary arteries supply deep white matter around the lateral ventricles. These originate from Anterior Cerebral Artery, Middle Cerebral Artery and Posterior Cerebral Artery. These arteries are present from the fourth month of gestation[135]. The basic circle of Willis is fully developed from 7 months of gestation. The medullary

arteries then end in a hypovascular zone in deep white matter. These penetrating arteries are accompanied by lateral branches, which are simple and less tortuous to begin with. Branching increases in conjunction with increased vessel diameter in line with increasing gestation[136].

The cerebral vasculature is not homogeneous and different areas have different degrees of vascularisation. This is most striking between grey and white matter, but also manifest within different regions within grey and white matter. The amount of vascularisation is dependent to a certain extent on the metabolic activity of a region[137].

In preterm brains the venous architecture is much less well developed so there are some relatively hypovascular areas. At more mature gestations the venous system is better developed. Thus there is less opportunity for haemorrhage and oedema and therefore less risk of venous infarction[138].

There is sparse vascularity in some areas predisposing them to ischaemic injury. These are found in border zones at the end of the penetrating arteries[59]. These areas respond poorly to reduced perfusion and increased metabolic demand. Injury is normally widespread in the preterm infant and more localised in more mature babies. This may be related to the development of perforating medullary arteries and the collateral circulation[94,139]. After 34 weeks gestation neovascularisation parallels the increase in brain volume[139].

Babies with PVL have less well developed architecture. In a post mortem microangiography study Takashima and colleagues demonstrated that brains with PVL had fewer vessels directly around the area of softening. These vessels were less patent due to surrounding oedema and haemorrhage. Within this cohort some infants appeared to have underdeveloped vasculature as the cause of their PVL as the infants had no clinical risk factors for developing it[135]. The blood vessels within the premature brain are less responsive to factors affecting their diameter. This lack of autoregulation means that at times of systemic hypoperfusion the brain is exposed to ischaemia. This results in the white matter being at an increased risk of injury. This effect is exaggerated in the sick infant when acidotic, shocked or hypocarbic for example[59].

The grey matter is much better vascularised than the white matter which probably explains why in spite of periods of hypotension and ischaemia, the white matter may suffer but the grey matter is well maintained[49].

Anatomy Of The Rodent Cerebral Vasculature

Blood vessels are found in the internal capsule through the later stages of rat gestation. At the time of birth the internal capsule is as vascularised as the nearby thalamus. The amount of lectin stained microglia decreases after day 12 of life, implying a slowing down of vascular development.

At birth there are basic penetrating vessels, which take on an arterial or venous characteristic by day 10. In the first week after birth the cortex rapidly expands and

the vessels grow to keep pace with this. In the superficial cortex sprouting is most prevalent within the first week of life. This process then proceeds into the middle cortex. Finally, to a much lesser extent, the vessel sprouting continues in the deep cortex [140].

By postnatal day 24 the vasculature is a complex network of penetrating vessels and capillaries. From this time point on there is no discernable difference in vasculature with increasing postnatal age[133].

OXYGEN AND ITS EFFECTS ON THE DEVELOPING RODENT BRAIN – A HISTOLOGICAL STUDY

Background

Retinopathy of prematurity is a disease process affecting the developing retinal vasculature in the premature infant. In the 1950s a link between oxygen usage and the development of ROP was found[141]. Since that time there has been much interest in the use of oxygen on the neonatal unit and its role in pathology as previously described in the introduction. The Edinburgh model of ROP, using a clinically relevant profile of variable oxygen described below, has been used to investigate various aspects of the pathogenesis of ROP. More recently the work has been extended to include an assessment of the brain injury sustained by pups reared in variable oxygen[142]. We know that ROP is a disease of disordered vascularisation and we also know that the rat pups in our model do experience some degree of white matter injury. The following study was set up to establish whether disorganised vascularisation within the rodent brain, akin to ROP, could be part of the explanation of this white matter injury.

Animal Models Of Brain Injury.

A range of animals is used for studying the effects of brain injury – sheep, piglets and rodents for example. They all have advantages and disadvantages. The choice of animal depends on what investigators want to examine. A recent survey concluded that rats are used in 54% of all neurodevelopmental research and mice are used in 39%[143]. As a result having an accurate way of comparing these species to human development is essential if experimental data is to be translated into clinical practice.

Our group uses the Sprague Dawley rat as this has proved to be an ideal species for retinopathy of prematurity work. The rat brain undergoes a growth spurt at 7 days, similar to that experienced by the human at the time of birth. Dobbing and Sands assessed brain weight gain as proportion of body weight gain to make this comparison. They assessed brain growth spurts in a range of species - guinea pig, rat, monkey, sheep, pig and rabbit. They managed to identify brain growth spurts in all species and could then categorise the animals into pre, peri or postnatal brain developers on the basis of timing of the growth spurt. They surmised that the time of maximal brain growth was also the time of maximal potential for injury. From this it was concluded that the 7 day old rat makes a good neurological model for the term infant. The authors themselves though comment on the heterogeneity of the brain and that the assumption of equivalent development in all areas of the brain may not be true[144].

It also has to be borne in mind that growth spurt timing alone may not reflect the actual developmental stage of the cortex and other cerebral structures. Using the spurt as a surrogate marker for cellular maturity may be inadequate. Romijn et al put forward a case for the 12-13 day old rat pup being equivalent to a term infant[145]. This would be extrapolated to mean that a newborn rat pup is a great model of extreme prematurity in the human.

Romijn's group assessed rat brain maturity on the basis of four factors: numbers of synapses, glutamate decarboxylase activity (related to the presence of GABA), choline acetyl transferase (related to the synthesis of acetyl choline) and the development of electrical activity.

They found a variation in conclusions between the four groups of data. However, the development of electrical activity is the best studied of all parameters. Prior to postnatal day 10 they describe only occasional bursts of electrical activity on the EEG of the rat pup. This may correlate with the high amplitude bursts described in infants of around 29 weeks gestation. The EEG of the rat then undergoes massive maturation over the course of the next few days from these infrequent bursts to a basic sleep wake cycling pattern. They described the EEG of a day 12-13 rat as showing "trace alternant" which is a pattern of quiet and active sleep. This trace is also seen in infants but only if they are at least 35 weeks gestation. From this they concluded that the rat neocortex at day 12-13 is most comparable with that of a term infant. They accepted that this may not be extrapolated to other regions of the brain but there is insufficient evidence to confirm or refute this suggestion[145]

Assessing other factors of brain development does demonstrate some regional variation along with gestation. There appears to be a window of increased sensitivity to glutamate toxicity in the rat at postnatal day 7-10. This equivalent time period in a human infant has been described in the hippocampus between 23-27 weeks gestation[145]. This is obviously behind the development of the EEG. As a result it is important to consider what area of the rat brain is being studied to decide on its relevance to the human preterm infant.

Clancy et al believe that brain development can be assessed in several different ways. Firstly looking at the morphological comparisons between species. This involves comparing for example the number of somites, neurons, synaptic development or size of the embryo. The drawback of this is knowing the exact date of conception so in the early few weeks the comparisons may be inaccurate. Equally when comparisons are made with human fetuses these are often made with tissue resulting from abortions / miscarriages and therefore are less likely to represent normal embryonic development[143].

The second method of assessment involves assessing susceptibility to injury patterns and growth spurts. This is based on the original work by Dobbing and Sands, as described earlier. They referred to these assessments as being "rules of thumb". This is on the basis that the research is several decades old and takes a very simplistic approach to calculating equivalence in neurodevelopment.

A website has been developed to help investigators gauge the equivalent gestations of various animal species to the human (translating time.net). This uses various physiological parameters in different brain regions and some statistical modeling to enable time points in a rat pup's life to be translated in to the equivalent for a human infant. The site enables comparisons to be made between species of differing gestations and gives equivalent gestations related to a selection of neuroanatomical parameters[143].

As our group was established primarily to assess the development of ROP in rat pups and the study of the white matter was secondary we did not have the luxury of changing species or time points for termination of the experiments. As a result we are making the assumption that the development of the rat hippocampus / internal capsule is in keeping with the cortex and as such this supports our use of the rat as a surrogate preterm infant.

The Edinburgh Model Of Retinopathy Of Prematurity

Utilising data from a unique computerised physiological monitoring system, the neonatal intensive care unit in Edinburgh has shown that premature infants who developed severe ROP, had significantly more variability in their transcutaneous oxygen levels when compared to infants who did not develop ROP[34]. Although the neonatal unit maintained all the infants within prescribed 'safe' oxygen limits, each patient experienced minute-by-minute fluctuations in their transcutaneous oxygen levels. These fluctuations, rather than the absolute levels of oxygen, were found to be the crucial element in the development of the disease. Following these findings, the group developed a laboratory rat model in which oxygen levels recorded in neonates have been translated into equivalent levels for administration to rat pups. Using this model, it has been possible to induce retinal changes in pups reared from days 1 to 14 after birth, similar to those in human ROP by using an atmosphere in which a computer recreated the oxygen variability recorded from a preterm infant who developed ROP[146,147,148].

Although there are guidelines suggesting limits within which the arterial oxygen levels for preterm infants receiving oxygen should be maintained, there is limited information on what constitutes normal levels for preterm infants[149,37]. Healthy preterm infants have mean arterial oxygen of 9kPa. In Edinburgh and elsewhere in the UK and US, neonatal units maintain preterm infants between 6 and 10 kPa (45-75 mm Hg) with a mean of 8 kPa (60 mm Hg). We have therefore taken 8kPa as our normal level (normoxia). A newborn rat breathing air (21% oxygen) has an arterial oxygen value of 12.9 kPa. In order to translate the recorded transcutaneous oxygen levels in infants to equivalent values for administration to rats, 4.9 kPa (difference between 12.9 and 8 kPa) was added to each data value recorded in the infant. This oxygen profile is administered to the rats as inspired oxygen. We have been able to do this because the relationship between inspired and arterial oxygen in rats is linear[150]. Because of the rapidly changing nature of the profile, and the difficulty of monitoring the arterial oxygen in the rat pups whilst they are in the isolator, we have not been able to ascertain in our model the relationship between inspired and arterial oxygen. Although this is a weakness in our model, the fact that we have been able to reproduce some of the retinal changes associated with infant ROP, confirms that the fluctuations in the profile are maintained to a large extent. In our experiments, rats exposed to the equivalent of the fluctuating oxygen profile at 8kPa, were calculated to have been hyperoxic 45% of the time and hypoxic 51% of the time (oxygen levels above or below 21%). The inspired oxygen levels in this paradigm ranged from 9.2% to 41.5%, and the computerised oxygen delivery system was able to effect a change in oxygen levels from 0.1% to 50% within one minute with a median difference between expected and achieved oxygen levels of only 0.3%[147].

Identification Of Blood Vessels Using Immunohistochemistry

Various antibodies have been used to identify blood vessels in a range of tissues. The three most commonly used markers (Lectin, Von Willebrands factor and CD 31) were tried in the rat brain to determine which should be used for this work.

Lectin

Our group, working on the Edinburgh model of ROP, has considerable experience in using lectin for studying blood vessels within the rat retina. FITC (fluorescein isothiocyanate) labelled lectin from Bandeirea simplicifolia has been used in these experiments. It has been an ideal marker for use with confocal microscopy, producing accurate and well defined images[148,148]. However the same immunohistochemistry protocol used on sections of the brain was not successful despite various changes to the protocol. For example, attempts were made to perfuse the pups with lectin and then simply cut sections of the brain and study them using confocal microscopy. Both large volumes of lectin (up to 25mls of 20µl/ ml) and high lectin concentration (1ml/ml) were used. The brains were cut into sections 60µm thick to enable some 3-dimensional appreciation of the vessels to be analysed. All of these techniques yielded disappointing results. It was felt that the perfusion technique might have been unsuccessful as many of the capillaries may not be patent at this stage. However, after reviewing the literature this does not seem to be a cause for concern in other studies[151,152].

Studies done comparing the use of FITC labelled Lycopersicon esculentum lectin and CD31 / PECAM-1 (platelet/endothelial cell adhesion molecule) in immunohistochemistry resulted in the CD31 delineating more vessels than the lectin. The overall vessel alignment was similar but the smaller details were picked out better using CD31 compared with lectin. There were also vessel sprouts that were not picked up by the lectin[153]. This is probably as CD31 is a specific endothelial cell marker whereas lectin binds to the vessel lining membrane.

Von Willebrands Factor

Von Willebrands Factor (VWF) is widely accepted as a marker of endothelial cells[154]. VWF is synthesised by endothelial cells. It causes adhesion of platelets to injured vessel walls and functions as a carrier and stabilizer of coagulation factor VIII.

CD31 and VWF have been evaluated concurrently to assess their specificity at delineating blood vessel development in rat mammary glands. This was done as it is accepted that CD31 is probably a more specific endothelial marker than VWF. This study involved a pre-treatment of both sets of specimens. Pre-treatment using trypsin was used for the VWF staining and heat treatment of the slides in a sodium citrate buffer used for the CD31 staining. This resulted in clear vessel marking, maintaining vessel morphological integrity and caused little tissue damage. However, the trypsin pre-treatment led to an increase in background staining and damage to the tissue sections being stained. The smaller vessels were easier to identify in the CD31 sections when compared to the VWF specimens. The mean and median vessel areas detected by both markers was the same, but CD31 picked out significantly more small vessels than VWF[155]. Immunohistochemistry was attempted using this

antibody but despite variations in protocol and significant alterations in concentration there was universally poor staining using this antibody.

CD31

CD31 / PECAM 1 (platelet / endothelial cell adhesion molecule) is part of a large family of cell surface glycoproteins that are essential in embryogenesis and development. It is found on the cell surface of platelets and leukocytes, in the cellular junctions between endothelial cells[156] and it is involved in modulating thrombosis[157]. Within the central nervous system its expression is confined to endothelial cells of the blood brain barrier[158]. Antibodies to CD31 interfere with these junctions between endothelial cells and affect the stability of the vasculature[156].

CD31 is expressed early on in life and sparse vasculature can be detected using CD31 antibody at day 3 in the rat cerebral cortex. The pattern of vessels becomes increasingly complex over time: expression of CD31 in normoxia peaks at day 13 in the rodent brain in relation to stabilisation of the vascular bed; after this protein expression appears to decrease. Under hypoxia the CD 31 response is both prolonged and increased. There is increased protein present by day 24, by which time the levels are up to three fold higher than they are in normoxic brains[133].

There is no agreement on the best endothelial marker to be used. CD 31 antibody is a reliable marker of endothelial cell differentiation[132]. It has been used extensively to study vessels in various types of pathology[159]. Successful staining for VWF and lectin is not guaranteed in normal vasculature and even less so in abnormal vasculature[153].Therefore, in setting a standard for the assessment of vasculature using immunohistochemistry, CD31 is the best antibody available and the one recommended by international consensus statements on the study of vascularity of tumours[160].

Methods

The study was conducted in accordance with a Home Office Animals (Scientific procedures) Act 1986 project licence. Pregnant Sprague Dawley dams were left to litter naturally and to clean both themselves and their litter. This is an essential stage of the bonding process between dam and pup. Care was taken not to disturb the pups too soon, as sometimes the dams would become agitated in the process of setting up the experiment and would eat their litter.

Along with the dam, the newborn rats were weighed and then put in an incubator and exposed to oxygen fluctuations in the method described previously. The oxygen profile fluctuated around a mean of 10 kPa for either 7 or 14 days. Control litters were exposed to room air only.

At either 7 or 14 days, the rat pups were given a terminal anaesthetic, the components of which were Xylazine hydrochloride (Rompun) 0.6ml and Ketamine (Vetalar) 0.3ml mixed with 0.9ml of phosphate buffered saline (PBS). Up to 0.2ml was administered intraperitoneally to each pup. Once anaesthetised, the pup's heart

was exposed in preparation for intracardiac perfusion and an incision was made in the liver to overcome the problems of venous congestion.

Tissue Preparation

Perfusion
The right ventricle was identified and PBS was infused. The volume used depended on the size of the animal. Perfusion was deemed to be adequate once the lungs appeared clear of blood. This involved the use of up to 10mls in the larger pups. If the brains were to be used for immunohistochemistry, this perfusion was followed with up to 5mls of 4% paraformaldehyde, again, according to the size of the animal.

[Note: In early experiments the brains were perfused with 0.5% PFA. This appeared to give adequate fixation of the retinas if they were left in fixative for a further two hours following sacrifice prior to dissection. But experience within the group suggested that the brains were not fixed well enough at this concentration. This became evident when cut on the freezing microtome. An extensive literature search was performed[133,131,120,161] and a colleague within Neuroscience in the university was consulted. In conclusion, the fixative of choice was deemed to be 4% paraformaldehyde.]

If the brains were to be kept for later RNA analysis they were not perfused with paraformaldehyde, but were perfused with PBS and then rapidly removed from the body and placed in vials on ice in preparation for dissection.

Brain Retrieval
The pup was decapitated and the scalp was peeled away from the skull. The sagittal suture was opened and the skull was dissected away from the cerebrum and brain stem, allowing the brain to be easily extracted from the vault. The brain was then either placed in 4% paraformaldehyde for fixing or was dissected immediately for storage and later RNA extraction.

Dissection Of Brain
The brains were dissected as soon as possible after death as they begin to soften quickly. The brain was dissected into specific areas, which were separately frozen for further RNA analysis. With the brain in the supine position any remaining spine was removed from the sample. The olfactory bulb was then removed from the frontal aspect of the brain followed by the hypothalamus, which is a spherical discrete piece of tissue on the inferior surface of the brain.

The brain stem was then peeled off the midbrain and cerebellum. The cerebellum is an easily identifiable entity and, following its removal, the midbrain was removed by cutting an inverted V from the base of the remaining cerebrum. The cerebrum was then laid out in its two halves. The hippocampus was identifiable as an oblong structure on each side of the midline. The striatum was collected next by gathering up the majority of tissue left apart from the cortex itself. The cortex was then rolled up and frozen. All samples were kept at -70 °C until needed.

Cutting The Brain

The brains for immunohistochemistry were left in paraformaldehyde from the day of sacrifice for 4-5 days to ensure adequate fixation. They were then placed in 30% sucrose for at least 24 hours to cryoprotect them before cutting. [The brains were not stored in sucrose for any longer due to concerns about the media becoming infected and the potential for tissue shrinkage in such a hypertonic solution].

The brain was then taken to the freezing microtome. Fixative was placed on the freezing plate along with a piece of filter paper and, once the fixative began to freeze, the brain was placed on top in the vertical plane. It was held in position until it had frozen to the plate. The temperature was reduced to -44°C and the brain was left for 30 minutes to ensure adequate freezing. The tissue was then allowed to rewarm to -32°C to -28°C and left to stabilise for 15 minutes. This was deemed the ideal cutting temperature producing the best quality sections when compared to other temperatures.

Brains were cut in to 40micron sections and placed in 12 well plates containing PBS for storage until needed for staining. The sections could remain like this in the fridge for up to 1 week.

Immunohistochemistry

The free-floating sections were stained with CD31 antibody. The protocol for which, is detailed in the appendix. Briefly, the sections were washed in PBS and placed in a swine blocking buffer then covered with primary antibody, [goat anti-CD31 (Santa Cruz)] and left in this overnight at room temperature. The sections were then exposed to a secondary antibody [polyclonal swine anti-goat (Dako)] for 2 hours before use of Strep ABC (Streptavidin Biotinylated Horseradish Peroxidase) and DAB (Diaminobenzidine). The sections were then mounted on to slides and counter stained with thionine in preparation for analysis.

Analysis

The sections were analysed using *Image pro, Stereology version 5.0.* Identical sections were examined for each animal. Three areas were identified within each section: internal capsule, hippocampus and cortex. Within these, random areas were selected for analysis by the software package. A calculation of the standard error of the mean was done to establish the number of areas needed to obtain the most accurate result (20 per area). This was done with the help of histology staff who did some initial counts prior to the main analysis and also advised on technique to ensure standardised analysis within the counting grid or with the caliper measurements. The blood vessels within these areas were assessed using two parameters: capillary density and capillary diameter. The analysis was undertaken by one investigator only, which does remove the problems associated with intra-observer error as what was being counted was standard throughout. But obviously this is potentially open to bias that may skew the results in favour of the hypothesis. This bias was partially removed by the investigator being blinded as to which tissue sections were from pups reared in room air or variable oxygen. The slides were simply labelled with a code (letter and date) at the time of staining and as such this was interpretable without the

key, which was kept separately on a computer database. This was only accessed at the time of data analysis.

Capillary Density
The vessels were counted using an x10 magnification field, so quantification of the amount of vasculature present could be made. A counting grid of 26 x 20 intersections was placed over the area to be assessed and a vessel was counted if it crossed the intersection. Vessels were not counted if they were on the perimeter of the section as they could theoretically be counted in two neighbouring sections.

Capillary Diameter
The diameters of the vessels were measured with x60 magnification. Random points were measured along the length of the vessel and only vessels with the appearance of a capillary were assessed.

From these numerical values formal statistical analysis of the blood vessels was possible using SPSS version 12.

Results

Demographic Data
All litters were standardised to between 10-12 pups. This should compensate for any degree of variation within litters. 5 litters reared in room air and 3 litters reared in oxygen have been analysed for this study.

Pup Weights
All pups involved in the experiments were weighed at birth and at the termination of the experiment on day 14. The pups reared in room air were significantly larger than the oxygen reared pups. The birth weight results shown are from the entire litter as it was impossible to label the pups at birth and follow individual weights through until termination of the experiment. The data listed in table 1 is for the entire litters of pups used in the experiment. The data in table 2 is for a smaller cohort of pups from different litters.

Table 1.
This table shows the birth weight and day 14 weights for the pups.

Mean (SD)	Room air n=12	Oxygen n=9	P value
Birth weight	5.5g (0.29)	5.84g (0.81)	0.39
Day 14 weight	28.3 (1.06)	25.3g (2.02)	0.01

This concurs with previous studies of this model.

Brain Weights

Table 2.

This table shows the brain weights for the pups at 14 days and what percentage of body weight that represented.

Mean (SD)	Room air n=8	Oxygen n=5	P value
Actual brain weight	1.07g (0.07)	1.02 (0.07)	0.26
% of body weight	4 (0.00)	4 (0.00)	0.75

There is significant room for error in the documentation of brain weight as this is reliant on dissection technique. However, as only one investigator harvested the tissue this should remove some of this bias. The reduction in numbers from the initial cohort is due to inaccurate recording of weights and so some data has been lost.

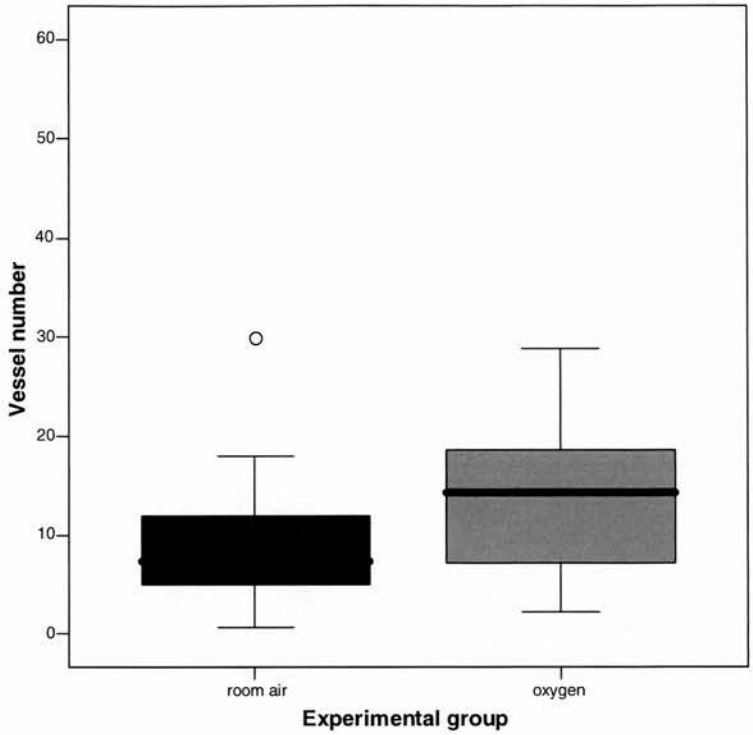
Capillary Count Data

The MannWhitney test was used to compare capillary counts between room air reared pups and those reared in variable oxygen.

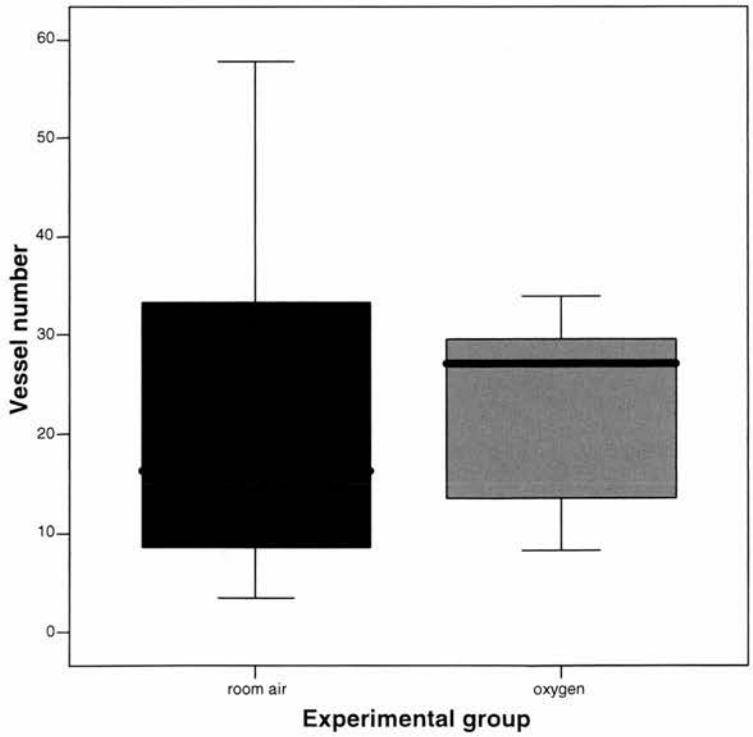
Table 3.

	Room air Median (IQR)	Variable oxygen Median (IQR)	P value
Cortex	7.36 (5.05-10.64) n=12	15.87 (12.04-20.69) n=9	0.20
Hippocampus	16.34 (9.48-31.55) n=10	27.08 (13.5-29.6) n=9	0.62
Internal Capsule	12.25 (3.85-24.59) n=11	9.38 (5.74-12.59) n=9	0.34

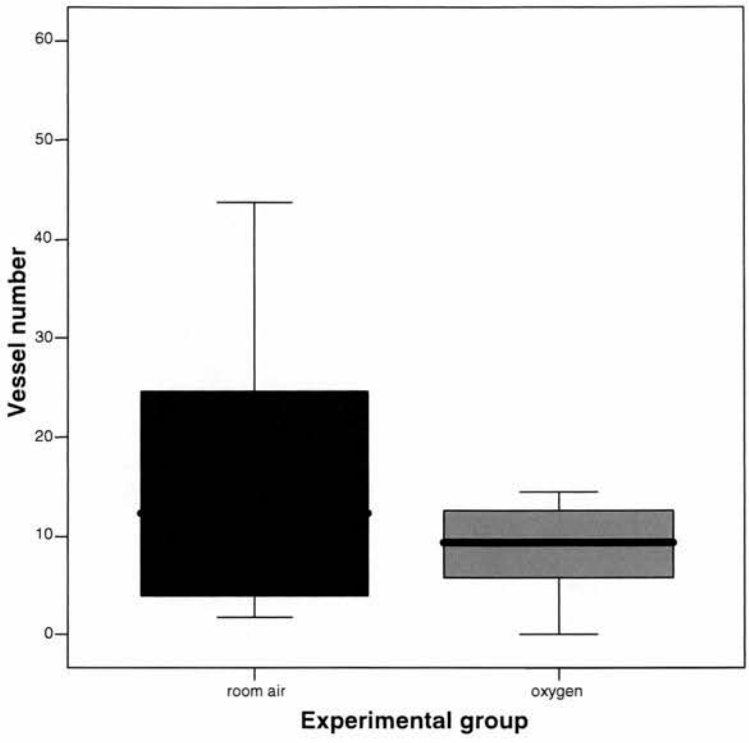
Graph 1.
This box plot compares the capillary counts within the cortex (p=0.201).



Graph 2.
The box plot compares capillary counts within the hippocampus (p=0.624).



Graph 3.
The next box plot compares capillary counts within the internal capsule (p=0.342).

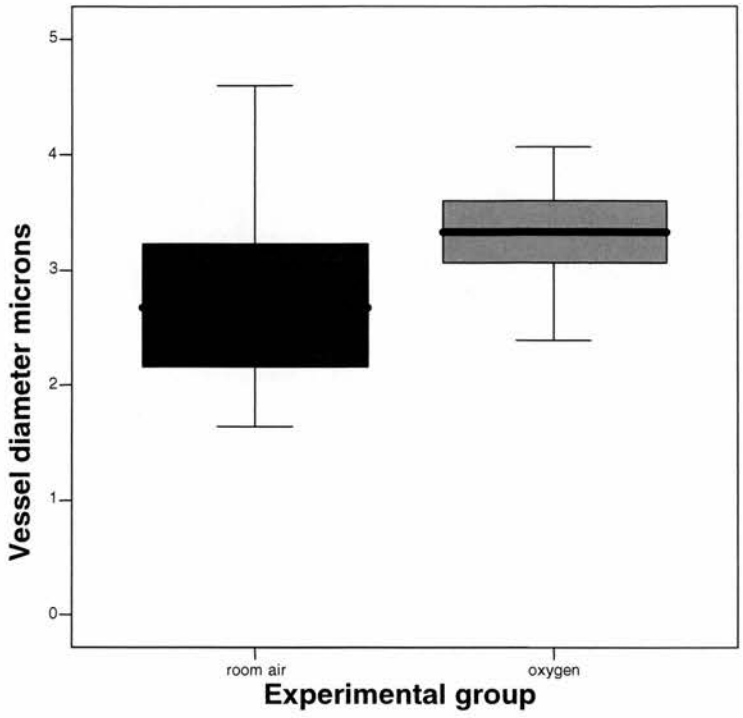


Capillary Diameters
The MannWhitney test was also used to compare capillary diameters between room air reared pups and those reared in variable oxygen.

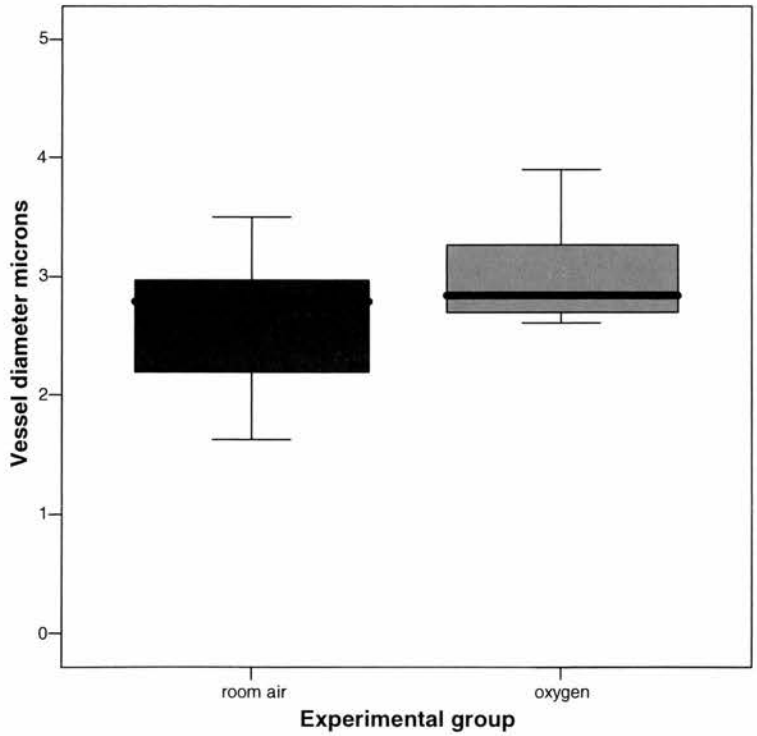
Table 4.

	Room air Median (IQR) microns	Variable oxygen Median (IQR) microns	P value
Cortex	2.68 (2.22-3.10) n=12	3.3 (3.05-3.52) n=9	0.055
Hippocampus	2.78 (2.26-2.96) n=10	2.84 (2.7-3.26) n=9	0.327
Internal Capsule	2.7 (2.36-2.99) n=11	2.84 (2.62-3.74) n=9	0.342

Graph 4.
The box plot below compares capillary diameters within the cortex (p=0.055).



Graph 5.
The next box plot compares capillary diameters within the hippocampus (p=0.327).



Graph 6.
This box plot compares capillary diameters within the internal capsule (p=0.342).

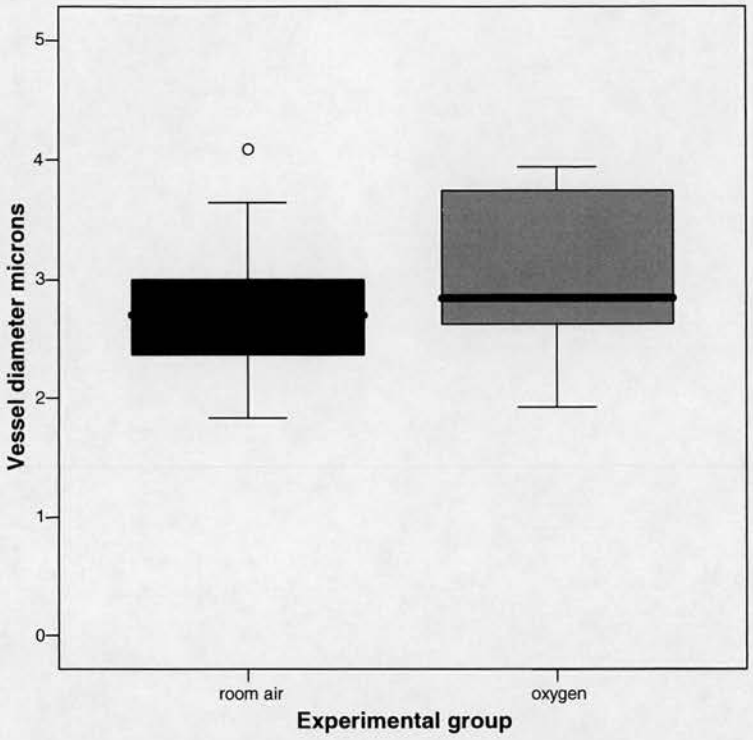


Figure 1.
CD31 staining of blood vessels in the rat cortex at x10 magnification.

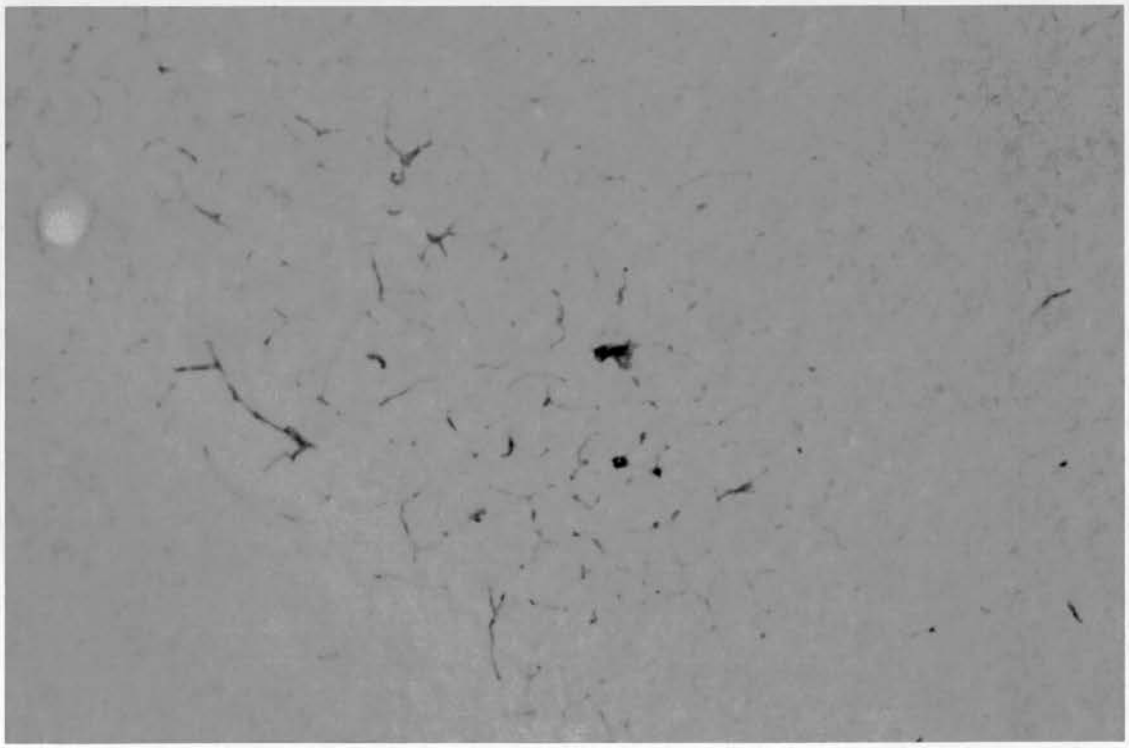


Figure 2.
CD31 staining of blood vessels in the lateral hippocampus at x10 magnification.

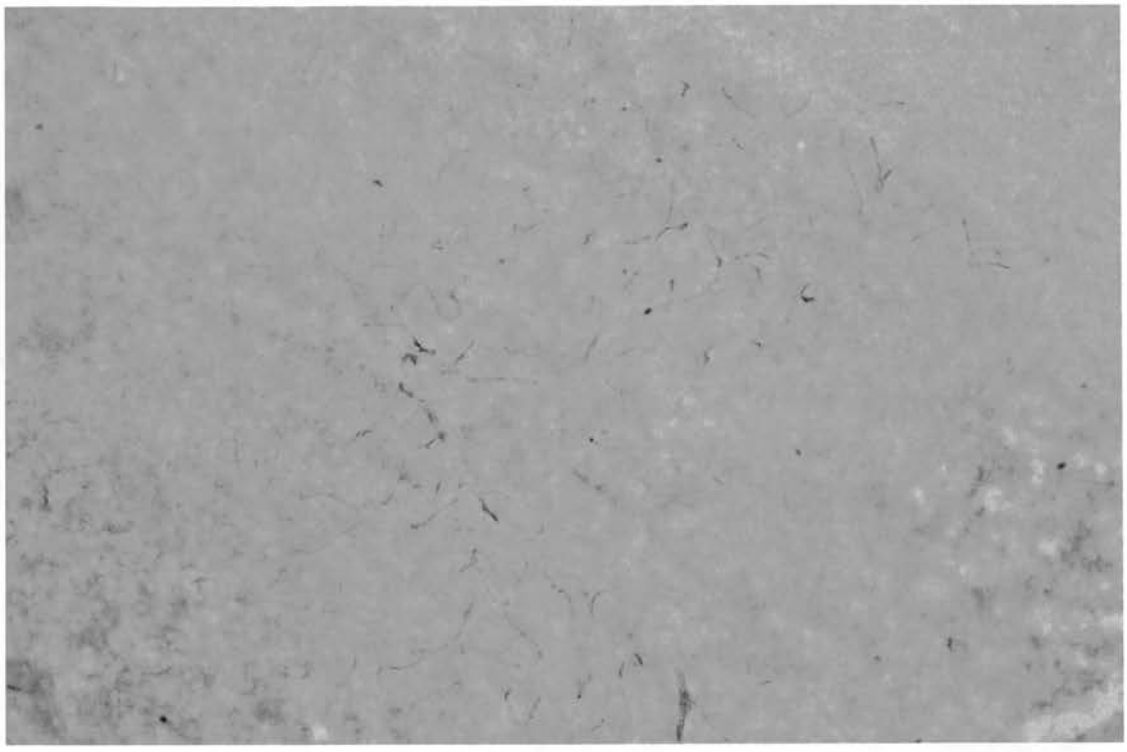
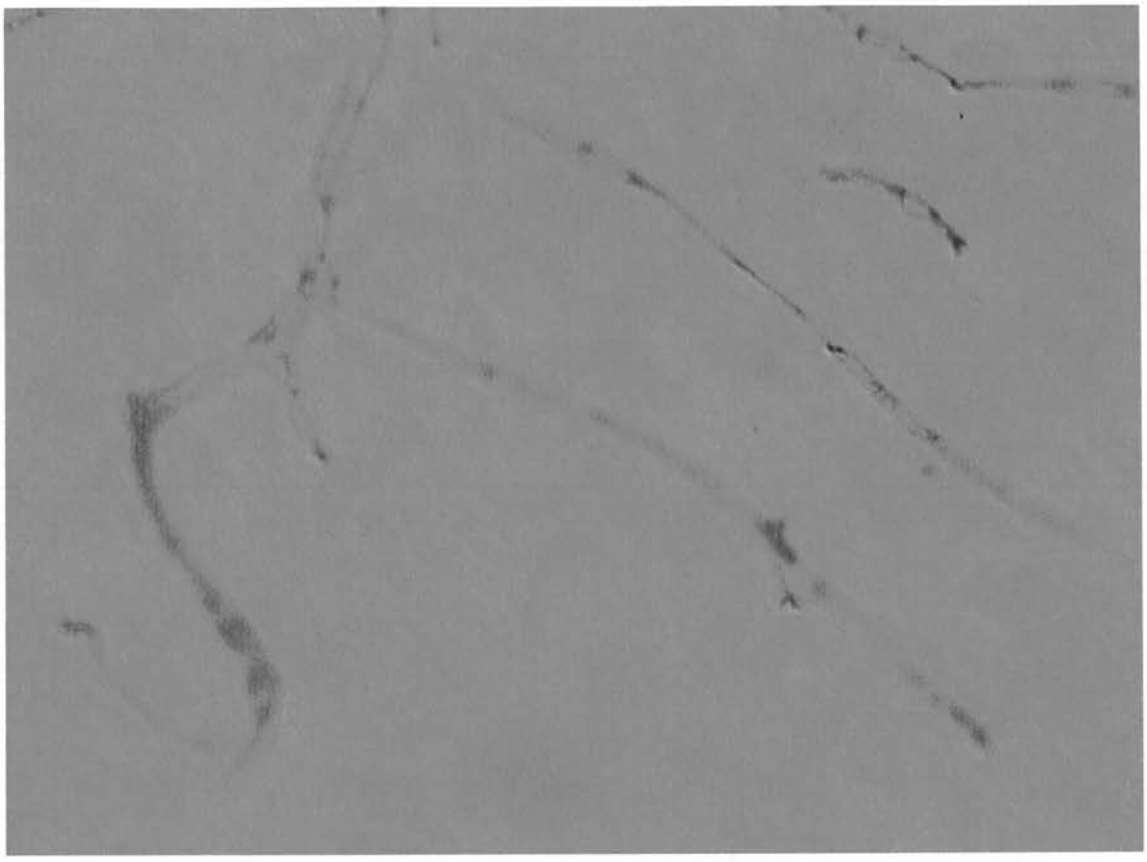


Figure 3.
CD31 staining of blood vessels in the hippocampus at x60 magnification.



Discussion

My results do not show a statistically significant difference between the group of pups reared in variable oxygen and those reared in room air; however there is a trend in the results showing that in the pups raised in variable oxygen the capillaries are more numerous and have a greater diameter.

These are similar results to those published in relation to hypoxic brain injury. Investigators have shown that following acute hypoxia there is simply an increase in blood flow and hyperventilation to compensate for the poor oxygenation[162]. If this hypoxia is more chronic then, along with an increase in polycythaemia, there are changes seen within the vasculature. These include an increase in capillary number and diameter at the site of injury[131]. It is shown that the local tissue response to the hypoxia is by increased expression of HIF1α and thus stimulation of vascular endothelial growth factor (VEGF). This leads to increased vascularity resulting in improved oxygenation. This is due to the shortened diffusion distance from capillary to cell [120,161,131].

HIF1α is expressed at times of hypoxia. It binds to the hypoxia response element within the cell nucleus and causes up-regulation of VEGF to increase blood vessel production[163]. VEGF is expressed by neuroectoderm and its receptors are found on invading endothelial cells. VEGF is found in increased amounts at times of active angiogenesis and is down-regulated when the process is quiescent. It is however, always present suggesting it has some role in maintenance of the blood vessels themselves[131].

It is widely accepted that between all the major vessels within the cerebrum there are areas of white matter that are not very well perfused. These are referred to as the border zones[164]. These areas are most susceptible to injury at times when brain perfusion is low. Poor development of capillary networks in these areas compounds the problem. This could be a factor contributing to the diffuse white matter injury seen in survivors of preterm birth.

In our model the pups are exposed to an excess of oxygen. With periods of relative hyperoxia there is less expression of HIF1α. This will lead to a reduction in angiogenesis with resulting tissue hypoxia leading to increased expression of HIF1α and increased VEGF with resultant increased angiogenesis. As the pups are exposed to regular fluctuations in hyperoxia / hypoxia, we hypothesise that this disrupts the process of cerebral angiogenesis in the same way that this mechanism causes ROP.

Hyperoxia causes vaso-obliteration by inducing apoptosis in endothelial cells, inhibiting endothelial cell proliferation and reducing the amount of VEGF available to maintain the vessels already present. Hyperoxia causes increased free radicals but hypoxia causes a reduction in free radical (NO) concentration resulting in vasoconstriction and thus a reduction in damage seen[165].

VEGF is a permeability factor and so we also surmise that altering its expression will affect the quality of the vessels produced and therefore also their function. We have

no information about astrocyte coverage of the capillaries, which is an important part of vessel maturation and essential for the regulation of the blood brain barrier. During vessel development the endothelial cells are initially covered by radial glia. These are then replaced by astrocytes providing more permanent stability[121,166]. Previous work from our group has shown that retinal vessels are less well covered by astrocytes when exposed to variable oxygen than when compared to room air (Wade – personal communication) and this might also be so in the brain. Extending this work to assess the astrocyte and pericyte coverage of the cerebral capillaries might also show this and therefore demonstrate a role for excessively permeable capillaries in white matter injury.

These results could also be an indication of free radical excess within the brains of these animals. Superoxide is a well-recognised vasoconstrictor. Nitric oxide is a vasodilator in low concentrations but at high concentrations is vasoconstrictive. NO is one of the molecules essential in the transcription of the gene for VEGF via HIF1α[163,163]. This has been shown in human glioblastoma and hepatoma cells. NO donors led to an increase in VEGF expression. This process is mediated through the HIF 1α pathway. This is one of the main mechanisms of angiogenesis in malignancy. But overall nitric oxides role in angiogenesis is directly dependent on the redox state of the cells involved[163].

The balance of nitric oxide being a vasodilator or vasoconstrictor is dependent on its role in the cyclo-oxygenase pathway. COX 2 (the inducible form of cyclo-oxygenase) can be activated by oxidant stress. Within the immature subject the exact effect exerted depends on the presence of the prostaglandin receptors and the binding of molecules within them. This can be variable leading to an altered response and an imbalance between vasoconstriction (PGE2 and PGF2α) and vasodilation (PGI2 and PGD2). At low concentrations free radicals result in vasodilation but at higher concentrations they result in vasoconstriction due to the activation of TXA2[167,168].

It could be that the variable oxygen is affecting the larger vessels within the brain and causing them to vasoconstrict, which in turn is leading to end organ hypoxia. This may explain why these results show a trend towards increased number and diameter, as seen in hypoxia, even though the animals are exposed to normoxia / hyperoxia. This is discussed in greater detail in the chapter on MRI as that study assessed the larger cerebral vessels.

Limitations Of The Study

Sample Size

The results show a trend in one direction, which does not reach statistical significance. This is quite possibly a type 2 error occurring because the number of pups used was small. In experiments like these it is important to use pups from as many litters as possible as there is inter-litter variation in terms of size, development, and anatomy. The size and growth of the pups is thought to be particularly due to variation in litter size. In our experiments this was minimised by using similar sized litters of 10-12 pups for all experiments. The results presented use pups from three litters per group. There are many factors that affect how many litters can be used in

this type of work. This work was carried out as part of a scientific group so other investigators needed pups of differing ages and diets. There were technical issues with the equipment that needed resolving. Some of our dams became distressed due to local building work and ate their litters. The study period was for two years only and each experiment takes four weeks to set up and run when it all runs smoothly. This does not allow for tissue processing and analysis, which was being done by one investigator alone (HS).

On the set up of the study a statistician was consulted to advise us of the sample size necessary to produce statistically significant results. As assessing cerebral vasculature was a new area of investigation calculations were made on the basis of expecting similar changes to those seen previously in the rat retinas. It was calculated that we would need 120 pups per group to produce reasonable results. This would involve the use of 10-12 litters in both variable oxygen and room air. Obviously in an ideal world we should have used this number as it would have enabled us to describe definitively if there was any effect on cerebral vasculature between the groups.

Unfortunately there were various factors that meant this was not possible. Firstly I was undertaking this work alone and only had a two year period to complete the study. The first year of study was hindered by technical difficulties with the equipment and so I only had twelve months in which to acquire the tissue and analyse it. Secondly, I was working as part of wider research group who posed other demands on the tissue acquired so experiments had to be coordinated between the retina team and myself. Thirdly, there is a requirement from the Home office to use as few animals as possible and so using a total of 240 pups is probably not ethically justifiable. The other side of that argument of course is that had we used the 240 pups we would have had a definitive answer and the experiments would not need repeating and so in the long run may lead to the use of fewer pups. The need for so many pups compared to other groups investigating hyperoxia is related to the fact that unlike many other models of retinopathy of prematurity our model does not inflict extreme hyperoxia on the animals and so the effects are more subtle requiring more animals to be studied to produce results that reach statistical significance. This is certainly a drawback of our model but it recreates the actual oxygen exposure experience by a preterm infant on the NNU more realistically than the extreme models.

Staining Technique

CD 31 is a well-used marker of endothelial cells: it stains not only patent vessels but also developing vessels. In this work it was found to be the best antibody to use. It proved to be very labour intensive and therefore reduced the number of brains available to assess. Perfusion of the brains with a fluorescent lectin could have been done to assess the vessels patency as lectin binds to the basal membrane and thus would not stain non-patent vessels. Perfusion using Evans blue would also have allowed us to assess the quality of the vessels. It is possible that the vessels from the pups reared in variable oxygen were more permeable as a sign of generally disordered angiogenesis. As other investigators were using other organs from these animals it was not possible to use these techniques as it would have affected their experiments.

Time Points

The pups used in this study were 14 days old. Unfortunately brain sections taken from 7 day old pups were all destroyed during the staining procedures because they were more friable than 14 day old brains. Despite altering the protocol in a number of different ways we were unable to obtain any for analysis: the brains were cut at different temperatures in case they were being cut at too low a temperature thus affecting their friability; the sections were mounted on slides and allowed to dry out prior to staining; different concentrations of hydrogen peroxide were tried as were different durations of DAB exposure as this seemed to be the stage when most sections were lost.

This was a huge shame as it would be fascinating to see how the vasculature is affected at earlier ages. If we had been able to study these brains we would have had greater insight in to how the vessels develop over time in particular whether there are greater changes earlier on which would imply a gestation related vulnerability, in the same way that white matter is more damaged at earlier gestations.

We would also have been able to assess the vasculature prior to the "growth spurt" described by Dobbing and Sands. It could well be that at earlier gestations the vasculature is so immature that there would be no discernable difference between the groups at this earlier gestation.

With the advent of magnetic resonance angiography techniques it may be possible to study the vasculature development as part of a longitudinal study. This would obviate the need to examine these brains histologically and enable study of the same rat through differing time points. We have scope within our Home Office Licence to perform these studies.

Conclusions

Our results are not statistically significant which is a shame as the hypothesis that the vasculature of the brain undergoes changes akin to ROP is highly plausible. They do show there is a trend towards abnormal vascularisation in the brains of rat pups reared in variable oxygen when compared to those reared in room air. These abnormalities include an increase in capillary number and an increase in vessel diameter. Both of these could be expected as a compensatory mechanism for potential end organ hypoxia seen at times of general hyperoxia.

MAGNETIC RESONANCE ANGIOGRAPHY OF THE RODENT CEREBRAL VASCULATURE

Background

Magnetic resonance imaging (MRI) is being used increasingly to assess preterm infants and try and identify infants at risk of long-term problems. There is a great deal of interest in the pathology known as DEHSI (Diffuse Excessive High Signal Intensity) seen on many infants' MRI images. This is believed to represent a more diffuse injury, not visible on ultrasound, that may manifest as the subtle injury seen in the survivors. It is believed to effect up to 80% of preterm survivors born at less than 30 weeks gestation. The changes are possibly related to sepsis or the presence of chronic lung disease. Both of these represent global insults to the developing brain[114,51,52].

Dyet et al showed a significant link between the amount of DEHSI present and developmental quotients at 18-36 months of corrected age. They showed an average DQ of 111 +/- 20 if there was no DEHSI on term equivalent scans compared with a DQ of 94 +/- 11.6 if there was some DEHSI and 92 +/-7.5 if the DEHSI was severe. There was no significant difference between the groups when comparing gestational age, birthweight, sex or age at term scan[51].

Interestingly DEHSI appears to exclusively effect cognitive development with no survivors in one cohort demonstrating any form of motor impairment[58]. There is much evidence to support the theory that white matter injury, typically PVL, is related to the blood supply to the brain. There are border zones within the parenchyma between the major vessels that are insufficiently perfused resulting in damage to that area[48].

Magnetic resonance angiography can be performed without the use of contrast to assess the cerebral vasculature using a technique called Time of Flight (TOF). In conventional MR imaging the pulsatility of the blood vessels causes artefacts. With TOF MRA it is different. The spins in the blood vessel continuously enter and leave the imaging frame, therefore, they are only subjected to a few radio frequency pulses. If a substance only experiences a few RF pulses their signal is high; If a substance experiences many pulses its signal is low. As a result tissues surrounding the blood vessels are low signal and hence why it is possible to get a good contrast between them and flowing blood. This sequence is dependent on the blood velocity. If the blood flow is too slow the signal will be reduced. As a result this technique can be affected by the clinical condition of the subject being scanned[169]. It also means it is difficult to visualise veins. The ideal TOF parameters include the shortest excitation time (TE) possible and low flip angle.

Use of contrast agent may mean that veins are easily visualised as well making analysis more complicated. Contrast agents such as gadolinium have been shown to have no effect on the clarity of the images acquired. However, they do reduce the time of acquisition, which may be of some benefit. Following the first pass though

they do diffuse in to the surrounding tissues therefore reducing the contrast between vessel and tissue. To overcome this a larger flip angel can be used[170].

Malametaniou et al have described a series of infants who were born preterm that underwent an MRI scan at term equivalent and compared them with term born controls. They used a TOF MRA 3 tesla protocol. Infants were excluded if they had any obvious form of white matter injury on MRI, ROP or perinatal stroke, as these would potentially affect the results obtained. They found that preterm survivors have a much less complex cerebral vascular network in terms of tortuosity when compared to control infants. They concluded that this might explain some of the problems these children have as they mature. This pattern was evident up to 18 months in a small cohort within the study who were imaged until this age. They concluded that the images were not just that of delayed maturation but a longstanding alteration in vessel development[171].

We wished to assess if there was any difference in the tortuosity or volume of the cerebral vessels of rat pups reared in variable oxygen when compared to those reared in room air.

Methods

Animals

The UK Home Office approved the study and all procedures were carried out according to the Animals (Scientific Procedures) Act 1986. Sprague-Dawley rats were used for this research. Once born, the experimental rat pups were weighed and then transferred to an incubator with their mothers. They were given time to acclimatize prior to starting the experiment. The control litters were reared in their usual cage for 14 days in room air. The experimental litters were exposed to the oxygen profile mentioned below with minute-by-minute variation in oxygen levels. Litters were standardised to 10-12 pups and variables such as diet and temperature were monitored and remained constant throughout. 6 rat pups were studied; 3 from control litters and 3 from experimental litters. Only this small number of pups were studied because this was a pilot study to see if the images could actually be obtained and effectively analysed. Due to time constraints no further pups were able to be studied.

Oxygen Profile And Delivery

In this study the oxygen profile and computer-controlled gas delivery system used have been described previously and are known as the Edinburgh model[148]. Briefly, transcutaneous arterial oxygen measurements were taken every minute for 14 days from an infant in the Edinburgh Neonatal Intensive Care Unit who went on to develop severe retinopathy. From this set of values, it was possible to derive the inspired oxygen in the rat needed to give the equivalent arterial oxygen levels every minute. Neonatal units within the UK maintain preterm infants at a mean arterial oxygen level of 8kPa. Rats breathing room air (21%oxygen) have a mean kPa of 12.9. Therefore 4.9kPa was added to each value obtained from the infant in order to translate it into a figure suitable for a rat[146]. In our experiments the animals were subjected to oxygen at an equivalent mean concentration of 10kPa with variability

added. This meant they were in a slightly hyperoxic environment. A computerised-controlled delivery system was used to inject oxygen and nitrogen into an animal incubator to mimic the oxygen profile over 14 days.

The pups used were part of the usual experimental protocols for ROP and brain studies within the group. Pups were chosen at random from those litters according to the availability of scanning time.

Anaesthesia

The rat pups were anaesthetized with 1-3% isoflurane. A temperature probe and respiratory rate monitor were attached. The animal was placed in the small-bore scanner whilst inhaling maintenance isoflurane, through a nose cone, to keep them sedated for the scan. Isoflurane was used because of its rapid reversibility. In the future it would be useful to allow the animals to recover so longitudinal studies can be carried out. At the end of these image acquisitions the pups were killed with a dose of intra-peritoneal pentabarbitol.

MRI Technique Used

We used a Varian 7T Direct Drive small bore imaging scanner. A time of flight (TOF) technique was used in this study. Simplified, this technique revolves around two principles:

1) The flowing blood is subjected to only a few radio frequency (RF) pulses before it is replaced by fresh blood in contrast to the stationary tissue surrounding the vessels which will experience a complete series of pulses[169].

2) The signal intensity of the RF pulses decreases throughout the series and eventually a low steady state signal intensity is reached[169]. Therefore; stationary tissues have low signal intensities and the flowing blood has a high signal intensity. The difference between them allows the vasculature to be seen without the invasive use of contrast media[169].

Parameters were set at the time of the scans and values were altered in order to achieve optimal images. As this was a pilot study, a range of parameters were used to try and establish the optimum protocol for future scanning. The parameters are listed below and exact values listed in Table 5.

TR = Relaxation Time

TE = Excitation Time

FOV = Field Of View

Resolution = The minimum size of objects that can be observed in an image. On our scanner this was calculated by taking the Field of View value (usually in mm) and dividing it by the Matrix Size.

Gain = The amount by which the original value of the signal received is multiplied. Both signal and noise are multiplied by that amount.

Averages = The number of individual scans of the same region that are accumulated and combined to form a single image. The same image is taken multiple times, one after the other and then combined into a

single image. The signal/noise ratio is higher for a multi-averaged scan than from a single average making the image clearer the more averages that are performed.

Slab = The volume of the sample/patient/subject/etc that is excited to produce images.

Flip Angle = The angle of the net magnetization vector in relation to the overall magnetic field.

RF Spoiling = The use of radiofrequency to transmit and receive at a certain phase. This removes some image artefacts by destroying magnetisation in the slab and thus stops it contributing to the signal received which would otherwise cause errors and artefacts during image reconstruction.

A survey scan was done initially to ensure the rat pup was in the correct position. For each rat pup approximately 5 scans were done each in order to get the best possible images. Each 3D scan took 15 minutes.

Table 5.

MRI Scanning Parameters

Rat	Coil	TR m/s	TE m/s	FOV mm	Resolution	Gain	Averages	Slab %	Flip angle °	RF Spoiling
1	Rapid 39	20	6.67	19.2x21.2x18	256x192x96	36	4	80	30	N
2	Rapid 39	20	3.17	28x21.2x25	256x128x192	30	8	200	30	N
3	Rapid 39	20	3.17	28x21.2x25	256x128x192	30	8	200	30	N
4	Rapid 39	20	3.58	28x28x28	192x128x128	12	4	125	30	N
5	Rapid 39	20	2.5	28x28x28	128x96x96	30	1	125	30	Y
6	Rapid 39	20	2.5	28x28x28	256x192x128	20	1	125	30	Y

Once the images were obtained they were accessed using *Image J* software and analysed using *Analyze 8.1* software. Firstly the rat brain was isolated from the whole image and then a threshold of intensity was chosen. This identified the areas with the highest signal intensities, i.e. the blood vessels (figure 1). Once the points picked out had been edited to delete artefacts and to add in vessels that had fallen below the minimum intensity threshold, a complete map of the main cerebral vessels was obtained (figure 2). This map was loaded into the tree analysis part of the software allowing both the tortuosity and the volume of 4 major vessels to be measured. These were the right and left middle cerebral arteries (MCAs), internal carotid arteries (ICAs), anterior cerebral arteries (ACAs) and the basilar arteries (BAs).

Tortuosity

Tortuosity was defined as a distance factor calculated by:

$$\frac{\text{Vessel length between defined end points}}{\text{Length of the straight line with the same end points}}$$

This 'distance factor' approach divides the total curve length by the distance between endpoints and is a straightforward initial measurement of tortuosity. More complex methods that take into account how often the curve changes direction could be used[172]. We kept to the more simple assessment of tortuosity as our vessels were not that complex. For each vessel an average length between 2 identical points was chosen and this same section was then used to compare all the rats.

Volume

For each vessel the average length of the section was the same. The number of voxels that made up the section in each of the vessels was determined. To determine the vessel volume this number was multiplied by the volume of one voxel ($0.007076mm^3$). All parts of the analysis were done blind with no knowledge of which pup was being analysed.

All measurements were done twice.

Figure 4.

Transverse slice through a rat head. The echogenic areas represent blood vessels within the head.

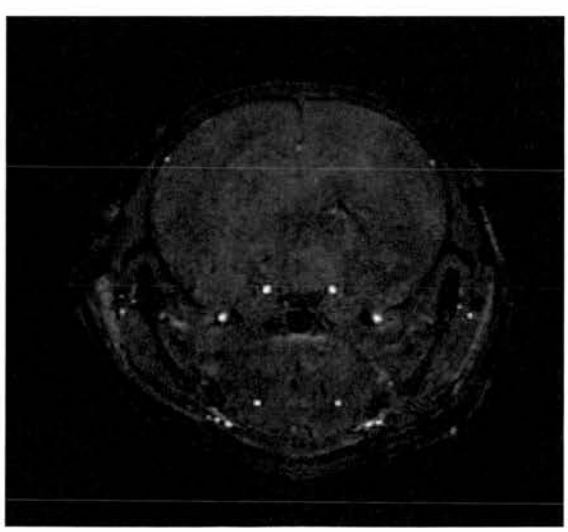
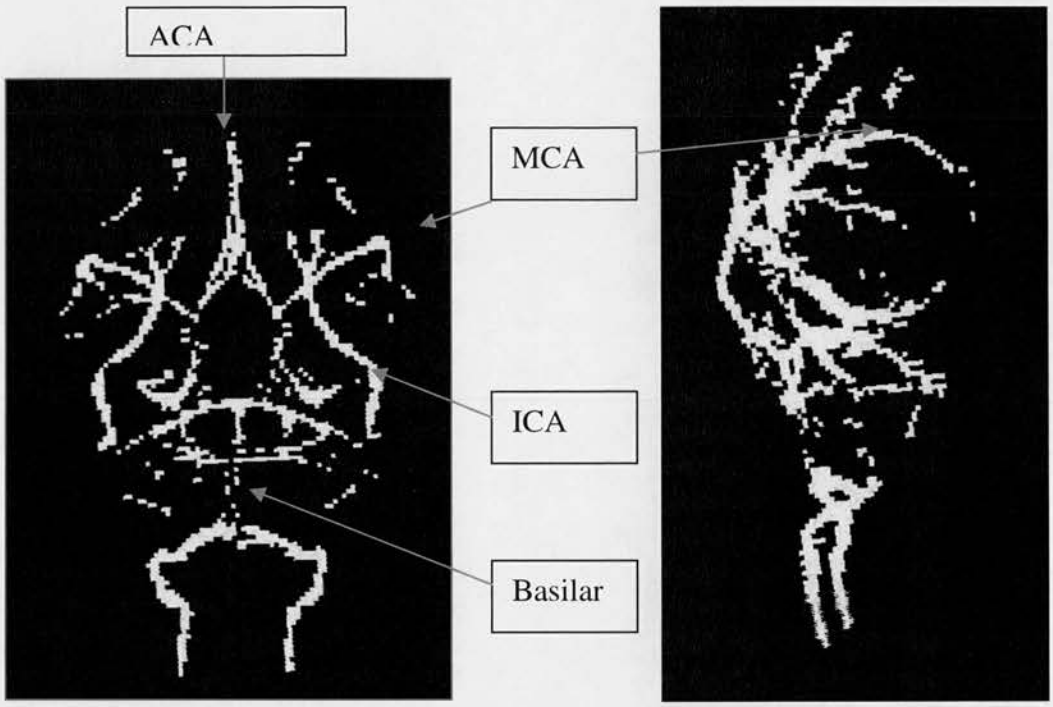


Figure 5.
Reconstructed images of the vascular tree in 2 differing planes.



Results

The following is data from the entire animal study I have completed. They are relevant here to show the differences in weights of the two groups of pups at the termination of the experiment and the relationship to their brain weight.

Pup Weights

All pups involved in the experiments were weighed at birth and at the termination of the experiment on day 14. The pups reared in room air were significantly larger than the oxygen reared pups. The birth weight results shown are from the entire litter as it was impossible to label the pups at birth and follow individual weights through until termination of the experiment.

Table 6.

Mean (SD)	Room air n=12	Oxygen n=9	P value
Birth weight	5.5g (0.29)	5.84g (0.81)	0.39
Day 14 weight	28.3 (1.06)	25.3g (2.02)	0.01

Brain Weights

The brain was weighed following removal from the skull and the results are presented here.

Table 7.

Mean (SD)	Room air n=8	Oxygen n=5	P value
Actual brain weight	1.07g (0.07)	1.02 (0.07)	0.26
% of body weight	0.04g (0.00)	0.04g (0.00)	0.75

Tortuosity

Analysis of tortuosity showed no statistically significant difference in the major vessels between the groups of rat pups.

Table 8.

Tortuosity of vessels of all rats exposed to either room air or variable oxygen. (DF = distance factor)

	Tortuosity DF		
	Room Air Mean (SD)	**Oxygen Mean (SD)**	**p value on t test**
ICA	1.05 (0.05)	1.11 (0.05)	0.26
MCA	1.03(0.02)	1.04(0.02)	0.46
ACA	1.05(0.02)	1.061(0.04)	0.52
Basilar	1.04(0.01)	1.05 (0.06)	0.74
Total	1.04 (0.00)	1.06 (0.03)	0.18

Volume

Table 9.

Volume of vessels of all rats exposed to either room air or variable oxygen

	Volume mm^3		
	Room air Mean (SD)	**Oxygen Mean (SD)**	**p value on t test**
ICA	1.16(0.51)	0.56(0.08)	0.13
MCA	0.69 (0.24)	0.41 (0.14)	0.07
ACA	0.99 (0.87)	0.57 (0.3)	0.45
Basilar	0.66 (0.39)	0.34 (0.29)	0.33
Total	0.88 (0.24)	0.47 (0.11)	0.02

Discussion

This study has used the technique of non-invasive MRA to reconstruct and analyse the blood vessels in the brains of 6 rat pups. We have shown there to be no difference in the tortuosity of the blood vessels but possibly a difference in the volumes of the blood vessels.

Our tortuosity results were disappointing, as we were hoping for some dramatic changes as seen by Malamateniou[171]. This is probably due to a number of factors. The main factor being that the cerebral vasculature of the rat is much less complex

than that of a human. With such a small volume of tissue (the dissected brain at 14 days weighs 1g) we are unable to visualise the smaller vessels of the rodent brain. This means that it is technically impossible to acquire the equivalent detail to the human studies. A future study could be done to assess these pups once they are fully developed adult rats. By this stage their brains would be larger and their vasculature may be more complex so greater differences would be visible. The ability to carry out a longitudinal study assessing the process of development of the vasculature is one of the advantages of MRA when compared to histological based studies.

Refining the imaging technique may also improve these results. A future study may benefit from the use of a contrast agent to assess smaller vessels within the vascular tree that we have been unable to visualise.

The volume results are more interesting. The rats reared in variable oxygen had smaller volume vessels. Their brain weights were comparable with the room air control pups, although their body weights were significantly smaller. From this it can be concluded that the smaller vessels are not simply a feature of a smaller brain. Clearly the brain weights could vary due to dissection technique and this has been alluded to earlier.

This effect is possibly the same pathological process that is seen in ROP. It has long been known that part of the development of ROP is hyperoxia induced vasoconstriction resulting in a hypoxic retina. Vasoconstriction is caused by the presence of free radicals in particular superoxide. In the immature brain there is little superoxide dismutase present to reduce these free radicals so they are able to exert their damaging effects. In this situation there is a fine balance between the presence of the vasoconstricting superoxide and the vasodilating nitric oxide[173].

The vasoconstriction causes up-regulation of HIF1α that increases expression of VEGF and therefore new vessel growth. We hypothesise that in the same way that minimal hyperoxia at clinically relevant levels can produce ROP[148] the same is happening within the rodent brain. As part of our laboratory studies it appears that within these rodent brains there is an increase in capillary number. This is presumably a response to the major vessel vasoconstriction leading to hypoxia within the white matter.

We are aware of the limitations of our study. The main factor being the small numbers of subjects used. We hope that this pilot data will be used to develop the technique further for a more in depth study. We have scope within our project licence to carry out longitudinal studies on the pups, which would give us a fascinating insight in to the process of vessel development. The reason for there being so few rat pups was due to time constraints. The analysis was undertaken by a BSc student who had one term only to complete this work. We decided it was better to keep the study to a manageable size so it could be completed within the allocated time rather than trying to do more but cut short the analysis. This work unfortunately could not be extended due to me moving away from Edinburgh and so it is left for other researchers to continue.

Isofluorane anaesthesia is known to reduce cardiac output and this could have an effect on the quality of the images acquired. High quality MRA images need a high velocity of blood flow[174]. It may well alter the size of vessels but this effect would also be evident in the room air control group.

The main advantage of animal experiments over clinical studies is that there are few in the way of confounding factors that may be affecting our results. There is recognized inter-litter variation that may explain our findings but otherwise both groups of animals are identical. The only factor different between the two groups is the use of variable oxygen.

Conclusion

In conclusion our pilot results contribute to the discussions regarding oxygen and its role in the development of diffuse white matter injury. We would like to develop this work not only to include more pups but also to perform longitudinal studies on the pups to see how the vasculature develops over time. We would also be able to image the pups both immediately after they are returned to room air and also allow them a recovery period in room air and see if this reverses the process in any way.

OXYGEN AND ITS EFFECTS ON THE DEVELOPING HUMAN BRAIN - A POSTMORTEM STUDY

Background

The Scottish Perinatal Neuropathology Study

This was a prospective population based study carried out between January 1996 and January 1999 in 22 Scottish centres. Its aim was to assess the amount and type of brain injury evident in a population of neonatal deaths and stillbirths. It included 745 perinatal deaths: 221 early neonatal deaths and 524 stillbirths. Within the neonatal death cohort clinical data was available for 137 infants; 88 consented to autopsy and 70 consented to having additional samples taken and stored for the study[96]. Within the stillborn cohort full clinical data and neuropathology was available for 191 infants[175].

Clinical information was gathered about all stillbirths and neonatal deaths according to a standard proforma. Information gathered related to maternal demographics, antenatal risk factors including pre-eclampsia, infection and anomaly scans. This was to ascertain whether there were antenatal factors that could have contributed to the brain injury seen. Further information was obtained regarding the labour, delivery and the neonatal course in the neonatal deaths.

Only neonatal deaths occurring in the first week of life were recruited as, within this time frame, it is deemed possible to describe with a greater degree of accuracy the timing of the brain injury. In the stillborn cohort the timing of last recorded heartbeat was ascertained to enable accurate timings of death and therefore gestation to be made.

70 of the 137 infants in the neonatal death cohort and 191 of 471 still born fetuses had a full post mortem including consent for retention of specimens for research. The post mortems were carried out in six different centres but standard blocks, up to 20, were taken and paraffin embedded for analysis and storage. It is from these that most of the samples for my cohort of neonatal deaths were obtained along with matched samples from the stillborn cohort.

The Scottish Perinatal Neuropathology study showed that 38% of term neonatal deaths and 52% of preterm deaths showed evidence of brain injury that predated the onset of labour[175,96,176] . The most obvious long standing prenatal injury included established cavitating infarcts, mineralisation and previous haemorrhage. They also assessed the brains for evidence of both white and grey matter gliosis which would suggest injury that was at least 3 days old. This is as opposed to neuronal eosinophilia and karyorrhexis or fresh haemorrhage and necrotic infarcts that suggest a more recent (less than 2 days) injury[175]. A judgement was made as to whether the presence of macrophage infiltration or extensive astrogliosis was likely to predate labour. It was acknowledged that this was most easy to identify in brains less than three days old. This injury was often not identified on ultrasound. Within the stillborn cohort 35% had evidence of brain injury that predated the onset of labour. The prevalence of brain damage was increased in infants born with indicators of birth

asphyxia suggesting a prolonged period of sub-optimal conditions in utero. In infants with birth asphyxia 57% demonstrated prenatal brain injury as compared to 8% in infants without asphyxia[96].
Cowan et al investigated the timing of brain injury in relation to neonatal encephalopathy using MRI images and where available post mortem. They studied over 300 infants of which 10% had post mortem and MRI images to compare. Their results showed that 80% of infants with hypoxic ischemic encephalopathy demonstrated acute injury without established white matter damage. There were 4 babies in the entire study who had damage that was felt to be antenatal in origin on MRI images and a further 3 who had small foci of gliosis on postmortem examination. They therefore concluded that most injury seen at the time of birth is perinatal in origin and very little predates the onset of labour[177].

There is clearly an argument to be had that post mortem is simply superior to MRI and therefore describes the incidence of brain injury more accurately. It appears to be generally accepted that MRI used in conjunction with traditional post mortem would be the ideal. In a study of 100 infants MRI data added information to the postmortem findings in 24 cases, but in 17 of these cases postmortem added vital information[178]. The study by Cowan et al used images obtained on both 1 tesla and 1.5 tesla scanners. With the increased power of todays scanners it may be that greater detail can be obtained in vivo and therefore increase the sensitivity of the MRI images closing the gap between histopathology and scan images.

Myelination And Myelin Basic Protein

Myelination
Neuropathological studies of fetuses and preterm infants must make allowance for the stage of development of the neuroanatomy. This includes both gross changes in structure such as the development of sulci and gyri and also the biochemical development, for example the presence of myelin[58].

Myelination is essential to the normal development of the nervous system. It commences in the peripheral nervous system at early gestations. The myelin formation here is related to the presence of Schwann cells and is a different process to the central myelination produced by oligodendrocytes.

Within the CNS, myelin is an extension of the oligodendroglial surface membrane and is composed of a bilayer of lipids held together by membrane proteins. Myelin basic protein (MBP) is one of these proteins making up 30% of the protein found. It is found at the cytoplasmic surface in the major dense line of the myelin sheath[179]. The presence of MBP has been shown to be 3-fold greater in the adult brain when compared to the brain of a newborn infant[180]. This is not surprising as areas of higher motor functions do not myelinate until some time after birth, some even decades later[181]. CNS myelination commences in the spinal cord and then gradually progresses to the cerebrum as gestation increases, continuing after birth until maturity.

Assessment Of Myelination

In terms of postnatal development, in 1987, Brody and Kinney, assessed myelination using Luxol fast blue to stain myelin tubules in 57 anatomical sites from within 162 brains from infants aged from birth at term to 33 months postconceptional age. They demonstrated how myelination progresses significantly in the postnatal period through different areas of the brain at differing time points. Their results provide the gold standard against which postnatal myelination can be assessed. It is acknowledged that by virtue of being an autopsy study these brains may not reflect what is actually going on in vivo[182]. Luxol fast blue staining detects myelin tubules and as such is useful in detecting mature myelin. Staining for MBP allows some assessment of the processes leading up to mature myelination and can be detected at earlier gestations than the myelin tubules positive for Luxol fast blue[181,183].

Myelination progresses gradually with the first signs in the spinal cord being seen at around the fourth month of gestation using immunohistochemistry for MBP [183]. In 1995, Tanaka et al, demonstrated that MBP is present around 1-6 weeks before Luxol fast blue staining becomes positive. They studied 66 brains and spinal cords from 14 weeks to 42 weeks gestation. No brain was positive for MBP or Luxol fast blue before 19 weeks. At 20 weeks MBP staining was present in the medial longitudinal fasciculus of the medulla oblongata. This was seen to gradually increase through gestation reaching maximal staining intensity by 34 weeks. Myelination was seen to follow just behind within the dorsal spinal roots and even further behind by two weeks in the ventral spinal roots. But the anterior cortico-spinal tracts did not appear to be fully myelinated until some time post term gestational age. They concluded that myelination progresses throughout gestation but at different rates in different areas of the central nervous system[184].

Periventricular Leukomalacia

Periventricular leukomalacia (PVL) is a disease process that may be focal (necrotic) or diffuse (gliotic)[58]. It is believed that the disease processes that lead to PVL of the focal variety lead to necrotic infarcts and then cysts. These cysts collapse to form local scars. Within the more diffuse variety of PVL it is now acknowledged that this process of damage is more widespread than previously thought. The pathological process leads to damage to all cell lines : astrocytes, oligodendrocyte precursors and axons. As a result gliosis is often seen in the presence of a reduction in myelination due to the oligodendrocyte damage[55]. It is this diffuse injury that we are more interested in and shall discuss below.

Oligodendrocyte Development

Oligodendrocytes originate from neural stem cells. Although essential in embryogenesis, there is an unknown number of stem cells that persist within the mature brain and these may contribute to repair at times of injury. The stem cells can differentiate into astrocytes or oligodendrocyte precursor cells (OPCs). These OPCs are present from the 12th postconceptional day within the spinal cord. These OPCs are affected by transcriptional factors Olig 1 and Olig 2. Olig 1 is essential for the survival and development of the OPCs and then becomes essential for myelinogenesis. Olig 2 is responsible for the motor neuron oligodendrocyte lineage.

As the OPCs progress into full mature oligodendrocytes they go through three further stages of development[58]:

The late oligodendrocyte progenitor (pre-OL) is the predominant cell type between 18-27 weeks of gestation with there being a few immature oligodendrocytes present. During this high-risk period for the development of PVL this cell type makes up 90% of the total oligodendrocyte lineage. These cells appear to be especially vulnerable to hypoxia and ischemia. There is evidence of significant apoptosis within these cells after such insults[58,185].
The immature oligodendrocytes make up an increasing proportion of the cell line at the later gestations (28-41 weeks). These cells are present in many areas within the brain. These immature cells generate pre-myelin tubules that begin to ensheath the axons. These immature oligodendrocytes are MBP negative. These MBP negative cells are present along with some MBP positive myelin tubules, found in the periventricular area from 30 weeks[185].

The mature oligodendrocyte appears at around 30 weeks when there is an associated increase in immature oligodendrocyte number. These mature oligodendrocytes are characterised by expression of myelin basic protein. The presence of MBP increases with increasing gestation. It is initially found in the deep white matter and gradually spreads through the cerebral hemispheres. These more mature cells appear to be more resistant to injury than their immature predecessors. This is most probably in part related to their increasingly developed antioxidant processes that in the less mature cells are easily overwhelmed and result in much of the white matter injury seen. The presence of these more mature cells signals the end of the significant period of white matter vulnerability[58].

Oxygen Related Damage
Of all the stages of oligodendrocyte development pre-OLs appear to be the preferential targets in diffuse white matter injury. They are susceptible to free radical, glutamate and cytokine toxicity[58]. The clinical evidence for this is that they are the main OL cell population at the time of maximal vulnerability to PVL. These pre-OLs preferentially die in diffuse white matter injury as demonstrated on TUNEL staining (a method of studying apoptosis based on labelling DNA strand breaks) and qualitatively there is loss of this cell population in brains where there is diffuse white matter injury. Pre-OLs are especially susceptible to free radical damage as they have reduced amounts of antioxidants, in particular glutathione, compared to the mature oligodendrocyte [58].

Gerstner et al demonstrated in a rodent model, both in vivo and in vitro, that pre-OLs are more sensitive to hyperoxia than mature oligodendrocytes. They showed that in vitro there was an increased amount of free radical injury in the pre-OLs exposed to hyperoxia (80% oxygen) when compared with the same cells in normoxia or the mature oligodendrocytes in hyperoxia. This was repeated in rat pups, aged 3,6 and 10 days, using an immunohistochemical study of MBP expression within the brain. In this study they demonstrated a reduction in MBP staining in the younger pups but not the more mature pups. This suggests that a maturation dependent process of cell

death is occurring in these brains. This is obviously very relevant to the clinical situation where the premature infant is taken from relative hypoxia, where it is developing normally in-utero, to relative hyperoxia, even in room air, at the time of birth when its ability to handle oxygen is limited due to immaturity[106].

Glial Fibrillary Acidic Protein And Reactive Astrocytosis

Astrocytes

Astrocytes are the predominant cell in the central nervous system (CNS) occupying 25-50% of the brain volume. They are part of the population of glial cells and are essential for development, migration and maintenance of neurons. Astrocytes communicate through large gap junctions allowing the passage of sugars, calcium and cAMP. Astrocytes are essential for the storage of glutamate, the brain's most important excitatory neurotransmitter. The astrocytes store glutamate and convert it to glutamine ready for transport to the pre-synaptic terminal of the neuron. Within the neuron it is changed back to glutamate, packaged into vesicles, and released as needed. Although glutamate is an essential excitatory neurotransmitter it is also harmful in excess. Modulation of the synthesis and storage of it is essential in maintaining brain homeostasis[186].

Astrocytes are key in producing lactate, which may act as a fuel for the neurons. They are also a good store of glycogen, again acting as a fuel for neurons. Astrocytes are essential for the support of neurites, which are outgrowing processes of neurons and both axons and dendrites. These astrocytes have a role in both development and repair following injury. They guide the extension of neurites and, after injury, the development of a glial scar restricts their extension[55].

Astrocytes are found migrating through the white matter throughout gestation. Few are found in the cortex. Their populations seem to be concentrated in the white matter at times of maximal vulnerability to development of PVL and then gradually decrease after 37 weeks gestation[54].

Glial Fibrillary Acidic Protein

Glial Fibrillary Acidic Protein (GFAP) is a structural protein found in intermediate filaments within the processes of glial cells, being generally considered a characteristic protein of astrocytes although it is also expressed in certain other cell types, such as non-CNS glial cells. GFAP occurs in more mature astrocytes and is not present in migrating cells [187]. As a result its expression varies throughout the brain. In the brain stem GFAP positive astrocytes have been identified from 15 weeks gestation, whereas the cortex apparently does not contain these mature cells until 30 weeks gestation[187,188]. Some evidence suggests that the number of GFAP positive astrocytes increases through later gestations[187].

An increased intensity of staining for GFAP and increased cell size suggests an up-regulation of these intermediate filaments and therefore an up-regulation in astrocyte function[188].

Brain Response To Injury

The response of the brain to different types of damage varies with gestation. An early histiocytic response is found from 12 weeks in the cavum septum pellucidum and is present in the leptomeninges from 20 weeks. As gestation progresses astrocytes develop a more mature reactive response and they become the main cells involved in damage and repair. After 6 months of gestation the responses of astrocytes are comparable to that of the mature human brain [189].

Myelination Gliosis

Myelination gliosis is a normal process that involves an increase in glial cells during myelination[189]. The glial cells are present in rows with small darkly stained nuclei with asymmetric cytoplasm. Bell et. al. showed that these cells are predominantly oligodendrocytes rather than astrocytes as they are negative for GFAP[176]. This phenomenon is not associated with white matter injury.

Reactive Astrocytosis

Astrocytes become reactive following a range of stimuli but in particular to inflammatory mediators related to ischemia and hypoxia[188]. This reaction is present within 12-48 hours of the injury being sustained. As a result there is an up regulation of GFAP expression, cellular hypertrophy, proliferation and process extension[188].

This process has both positive and negative effects on the tissue. It may be part of the repair mechanisms instigated following injury due to the astrocytes secretion of transforming growth factor (TGF) - β and thus help repair the tissue. Astrocytes are also known to secrete vascular endothelial growth factor (VEGF) which promotes neovascularisation around the site of injury[190,191].

However, the reactive astrocytes may also contribute to the injury through their ability to generate free radicals. In particular they have been found to secrete inducible nitric oxide synthase (iNOS) and produce nitric oxide (NO). As astrocytes communicate through large gap junctions these also allow for the transport of these damaging free radicals and therefore extension of the injury. The free radicals cause uncoupling of these gap junctions, which disrupts the communication between astrocytes and neurons resulting in increased injury. Paradoxically, astrocytes are a store for large amounts of antioxidants,especially glutathione[192].

Astrocytes also secrete inflammatory mediators such as Tumour Necrosis Factor (TNF)α which is directly damaging to oligodendrocytes[176]. They may also secrete inhibitory molecules that stop axon regeneration. Nuclear factor κ B (NFκB) is secreted by astrocytes and has both a protective and detrimental role to the developing brain. The secretion of NFκB leads to an increase in the presence of neurotrophins, which in turn increases neuronal survival. NFκB also prevents astrocytic apoptosis. Unfortunately this is balanced against the more negative effects it exerts including inhibiting neurite outgrowth. In vitro studies have shown that inhibition of NFκB is actually neuroprotective[193].

Many proinflammatory cytokines are increased in reactive astrocytosis. Exactly which ones exert what effect is difficult to elucidate. It is acknowledged that the

developing brain has relatively immature and variable immune responses. Some of these cytokines are present as they have passed through the blood brain barrier but some are endogenous to the brain tissue. Maslinska et al showed that, in a rodent model of premature asphyxial injury, the most important endogenous cytokine was IL-15. This is not only an important growth /differentiation factor but also may aid proliferation of glial cells leading to gliosis and axonal regeneration[194].

Following injury astrocytes change their morphology and function; they no longer provide neurons with fuel. From a practical point of view they migrate at the time of injury often resulting in direct physical trauma to the neurites they were attached to. This results in cyst formation due to movement of the astrocytes and death of the neurons as they are no longer supported by the astrocytes[195]. Glial scars, or areas of gliosis, around the injury may limit the damage sustained by protecting the brain around the site of injury from the inhibitory factors secreted by the astrocytes involved in the damage[194].

It is generally believed, though in the human brain the evidence is not directly available, that astrocytic changes become prominent only 12-48 hours after the insult[55]. In the NCPP (National Collaborative Perinatal Project) population these changes were present in 9% of brains at mid-gestation post-mortem but had increased to 59% at term [189]. This is in keeping with the gradual maturation of astrocytes through the later gestations increasing their ability to express GFAP. The long-term effect of this is widespread gliosis and under-myelinated white matter, with some loss of grey matter volume apparent on neuro-imaging studies[189]. The process of development of PVL involves a period of necrotic cell death. This occurs to all cell types including axons, astrocytes and oligodendrocyte precursors, which explains the wide-ranging effects of this type of injury[55].

It appears that these first few days after injury are the crucial time for development of gliosis and that after this time there is little ongoing formation of reactive astrocytes[196].

The following body of work is an extension of the Scottish perinatal neuropathology study, specifically assessing whether the white matter injury present is different between the brains of still born infants or those who have suffered a neonatal death. We hypothesise that if there is a difference then this might be related to some postnatal factor experienced by the live born infants only.

Methods

Identification Of The Cases.

The study was set up to compare the neuropathology in stillborn and liveborn infants. Neonatal deaths that had a post mortem performed were identified from the computerised records of all infants treated at the neonatal unit in Edinburgh. It was decided to use local infants, as it would be easier to obtain clinical information about them, in particular monitoring data was available from our cot side monitoring system. It was ascertained by consultation with the clinical notes whether the post-mortem consent also allowed for use of the tissues for research purposes. Once this

cohort had been identified a matched group of infants were chosen from the cohort of still births collected for the Scottish neonatal neuropathology study described earlier[96]. The infants were matched on the basis of sex, gestation and weight.

Finding The Blocks

The paraffin blocks were then identified from the long-term storage facility at the Western General Hospital. The temporal lobe and basal ganglia blocks were identified where possible. These areas were chosen as they were felt to be the areas most likely to demonstrate damage that could be attributed to postnatal oxygen administration. 5 microns thick sections were cut by microtome, placed in a water bath and mounted directly on to Superfrost slides. These were left to dry overnight in an incubator.

Initially, haematoxylin and eosin staining was performed to confirm that the blocks to be studied were of the correct areas. Once the correct blocks had been identified the necessary sections were cut.

Staining For MBP

The slides were deparaffinised in alcohol then antigen retrieval was performed using Citric acid pH6. Endogenous peroxidases were blocked with 3% hydrogen peroxide. They were exposed to normal swine serum (NSS) then the primary antibody (MBP 1:1000 in NSS). The negative control slides were simply exposed to more NSS.

Following a wash in TBS the slides were exposed to the secondary antibody, biotinylated anti-rabbit immunoglobulin (SARBO) then Streptavidin Biotinylated horseradish peroxidase (Strept ABC). They were then treated with Diaminobenzidine (DAB) and counterstained using haematoxylin. (Further details of methods are described in appendix 7.). All sections were stained within the same day to ensure standardisation of solutions and conditions.

Staining For GFAP

The slides were treated with trypsin and then exposed to NSS followed by the primary GFAP antibody at a concentration of 1:2000. The negative control slides were exposed to NSS only. Then SARBO, washed in TBS and finally exposed to StrepABC. The slides were then exposed to DAB and counterstained using haematoxylin. (Further details of methods described in appendix 8.). All sections were stained within the same day to ensure standardisation of solutions and conditions.

Analysis

MBP

Myelin basic protein was difficult to analyse as staining was very localised in early gestations and its expression was variable with gestation (see discussion). The same area of hippocampus and basal ganglia was identified anatomically in each section and a visual score of 0-5 was given for this area. Both Professor Jeanne Bell (JB) and myself (HS) undertook this analysis independently. We then met and reviewed the sections to ascertain our level of agreement on the quantity of staining present.

The same area was then identified and its density of staining determined using *Image pro* software. This was done by setting a threshold of intensity of staining to quantify within each region. All methods have been analysed. Examples of the sections analysed are shown below in figures 6 and 7.

GFAP

Professor Bell and myself again independently scored these sections on a scale of 0-5. As this staining was more generalised within the section a range of areas were selected and analysed and an average for the section was taken. The number of areas varied from 5-10 depending on the size of section and the amount of white matter present. Again a consensus was reached regarding our individual scores.

Image pro was used to quantify the amount of staining present. Both methods have been analysed. An example of the section analysed is shown below in figure 8.

Figure 6.
Myelin basic protein staining visualised at x10 magnification.

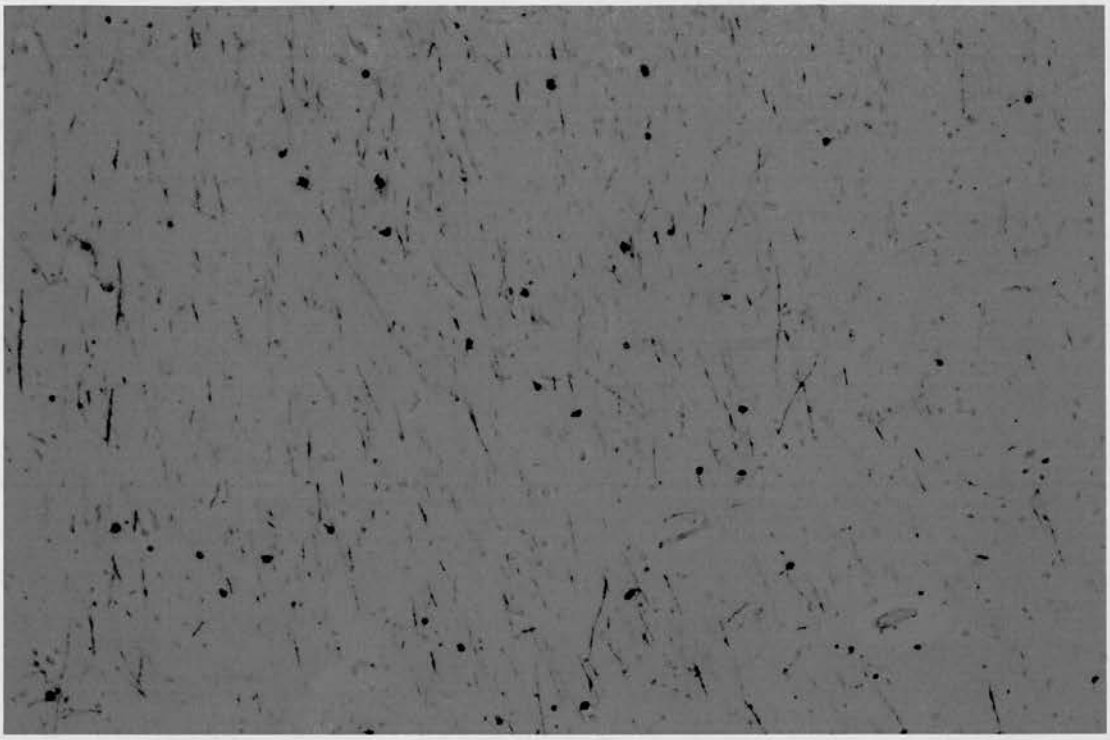


Figure 7.
Myelin basic protein staining visualised at x40 magnification demonstrating myelin fibrils from the oligodendrocytes.

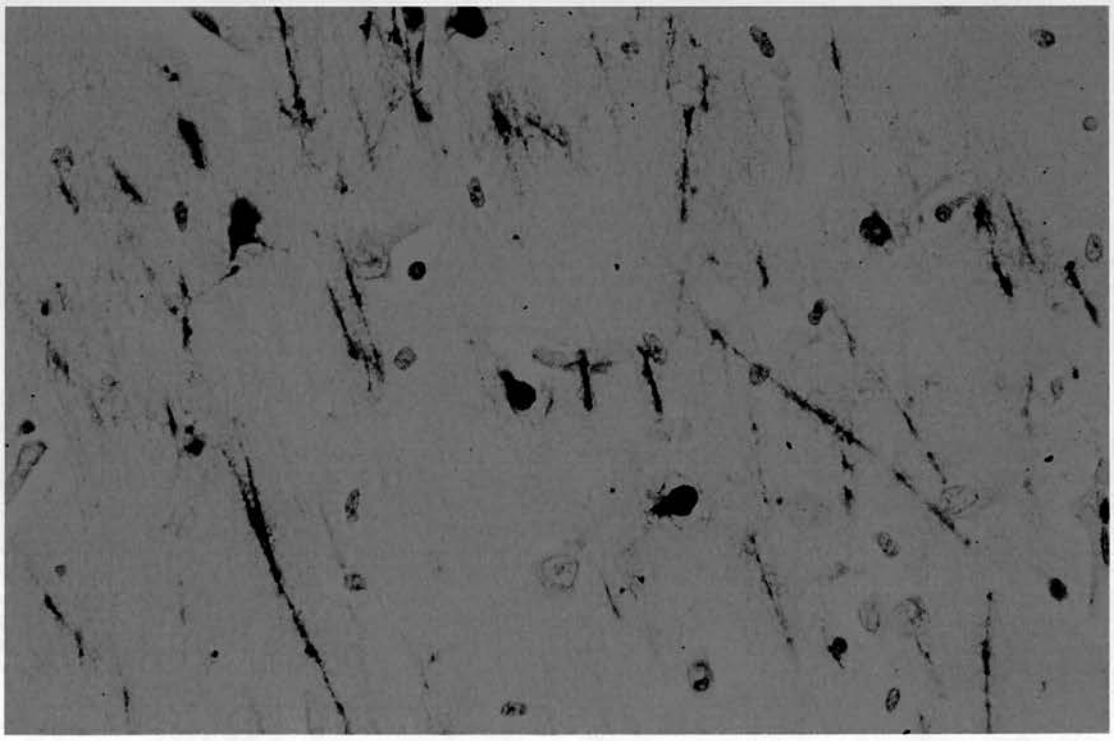
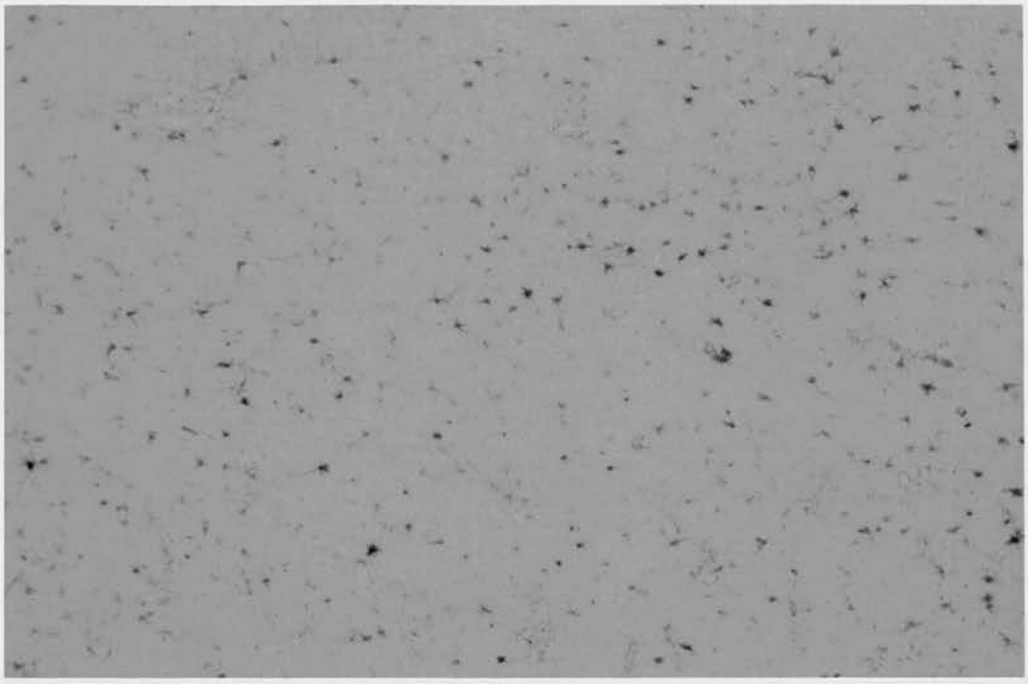


Figure 8.
Glial fibrillary acidic protein staining of astrocytes.



Results

Basic Demographic Detail Of The Cohort
Neonatal deaths (NND) were identified from the Scottish Perinatal Neuropathology study (SPNS) and local hospital records as previously described. These were then matched for gestation, birth weight and sex with a cohort of stillbirths (SB), also from the SPNS.

Table 10.
Demographics of the cohort used for the study.

	NND	SB	P value
Number	28	24	
Sex M : F	10:18	11:13	0.116
Gestation weeks, median (IQR)	36 (31-40)	36 (27.25-39.75)	0.5
Weight g, median (IQR)	2515 (1876-3064)	2035 (937-3147)	0.267

The groups were well matched as shown.

Table 11.
The table below demonstrates number of cases used in each gestational age range in this study.

Gestation in weeks	22-24	25-27	28-30	31-33	34-36	37-39	40-42
Number of cases	1	8	4	6	12	7	14

From within these groups, sections were cut and stained from blocks that were identified to contain basal ganglia and hippocampal material.

Table 12.
Number of cases used in each part of the study.

	NND		SB	
	Basal ganglia	Hippocampus	Basal ganglia	Hippocampus
MBP	21	11	21	13
GFAP	23	11	19	11

The discrepancy between the final and the original case numbers is mainly due blocks from some cases not having anatomical areas available or identifiable. In addition some sections were damaged during processing.

Myelin Basic Protein Results

Table 13.
The table below demonstrates the number of cases used in each gestational age range in this part of the study.

Gestation in weeks	22-24	25-27	28-30	31-33	34-36	37-39	40-42
Number of cases	1	8	3	5	11	7	14

Weighted Kappa Value

The scores assigned to each slide by JB and HS were assessed for intra-observer error. The weighted kappa value was calculated to be 0.68 (very good agreement).

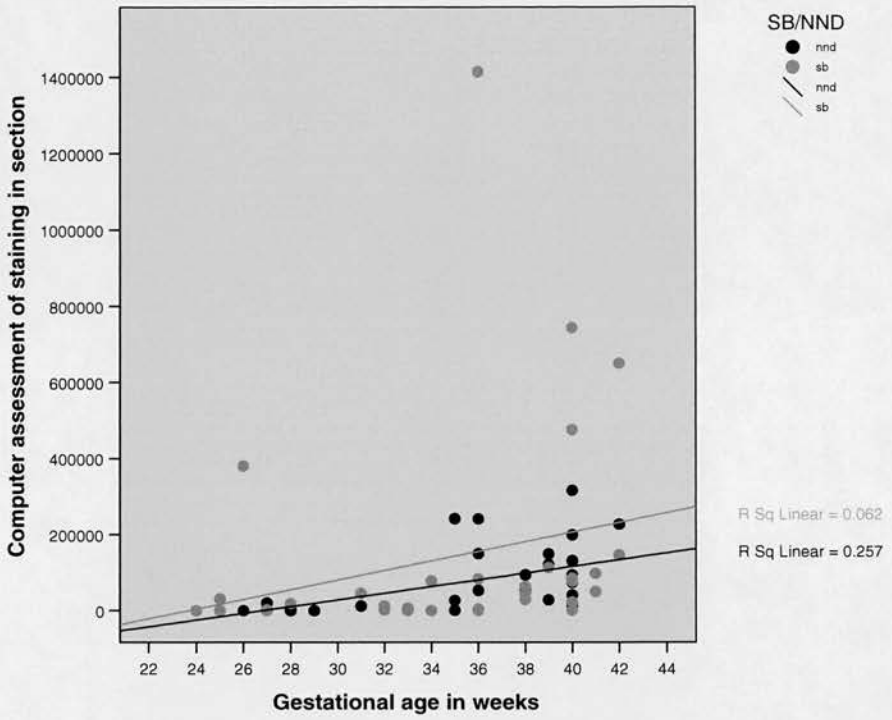
Spearman's Correlation Of Quantity Of MBP Staining In Relation To Change In Gestation.

Table 14.
Spearman's correlation coefficients of MBP staining with gestation.
Significant P values (<0.05) are shown in bold.

	Total	NND	SB	BG	HC	NND		SB	
						BG	HC	BG	HC
Computer	**0.615***	**0.618***	**0.584***	**0.516***	**0.803***	**0.556****	**0.761****	**0.48*****	**0.76****
JB	**0.575***	**0.667***	**0.459****	**0.582***	**0.673***	**0.699***	**0.688****	**0.459*****	0.537
HS	**0.394***	**0.505****	0.283	**0.476***	**0.519****	**0.65***	**0.764*****	0.303	0.387

* = p=<0.001
** = p=0.01-0.001
*** = p=0.05-0.01

Graph 7.
This scatterplot shows the quantity of MBP staining judged by computer analysis plotted against gestation.



Comparison Of Quantity Of MBP Staining Between NND And SB Brains.

Table 15.
Comparison of quantity of MBP staining between NND and SB (p values using Mann Whitney U test) using computer data.

	NND Median (IQR)	SB Median (IQR)	P Value
Total cases	46739 (631-130474)	30225 (1208-84759)	0.737
BG	52627 (9972-149644)	45509 (11953 – 85271)	0.95
HC	40850 (0-79346)	2339 (0-83224)	0.929

Table 16.
Comparison of quantity of MBP staining between NND and SB (p values using Mann Whitney U test) using JB data.

	NND Median (IQR)	SB Median (IQR)	P Value
Total cases	2 (0.25-3)	1.5 (1-3)	0.631
BG	2 (1-3)	2 (1-3)	0.896
HC	1.5 (0-2)	1 (0-1.5)	0.491

Table 17.
Comparison of quantity of MBP staining between NND and SB (p values using Mann Whitney U test) using HS data.

	NND Median (IQR)	SB Median (IQR)	P Value
Total cases	1.25 (0-3)	0 (0-3)	0.354
BG	3 (0.5-4)	3 (0-3)	0.387
HC	0 (0-1)	0 (0-0)	0.265

Comparison Of MBP Staining Between NND Brains Which Were Documented To Have Some Injury That Predated Delivery And Those That Had Injury That Was Definitely Only Postnatal In Origin.

Nine cases out of 21were available for the basal ganglia study and 5 cases out of 11 for the hippocampus study. The Mann Whitney U test was used to compare groups. No comparison shows a significant difference.

Table 18.
Comparison of quantity of MBP staining between cases with no prenatal damage and those with evidence of prenatal damage (p values using Mann Whitney U test) using computer data.

	No prenatal damage Median (IQR)	Prenatal damage Median (IQR)	P Value
Total cases	46739 (12847-127956)	46727 (0-122128)	0.737
BG	39775 (7690-149444)	93709 (11762-227126)	0.775
HC	46834 (31248-76905)	0 (0-73758)	0.400

Table 19.
Comparison of quantity of MBP staining between cases with no prenatal damage and those with evidence of prenatal damage (p values using Mann Whitney U test) using JB data.

	No prenatal damage Median (IQR)	Prenatal damage Median (IQR)	P Value
Total cases	2 (1.38-3)	1.5 (1-3)	0.631
BG	2.5 (1.25-3)	2 (0.5-3)	0.796
HC	2 (1.13-2.63)	0 (0-1.5)	0.073

Table 20.
Comparison of quantity of MBP staining between cases with no prenatal damage and those with evidence of prenatal damage (p values using Mann Whitney U test) using HS data.

	No prenatal damage Median (IQR)	Prenatal damage Median (IQR)	P Value
Total cases	1.5 (0-3)	1.25 (0-3.438)	0.354
BG	3 (0.5-3.75)	3 (0.5-4.5)	0.490
HC	0 (0-1)	0 (0-0.75)	0.816

Glial Fibrillary Acidic Protein

Table 21.
The table below demonstrates number of cases used in this study for each gestational age range.

Gestation in weeks	22-24	25-27	28-30	31-33	34-36	37-39	40-42
Number of cases	1	7	3	6	10	7	13

Weighted Kappa Value
The scores assigned to each slide by JB and HS were compared . The kappa value of 0.83 showed good agreement.

Spearman's Correlation Of Quantity Of GFAP Staining In Relation To Change In Gestation.

Table 22.
Spearman's correlation of gestation vs quantity of staining present.
Significant p values ($p<0.05$) are shown in bold.

	Total sections	NND	SB	Total		NND		SB	
				BG	HC	BG	HC	BG	HC
Computer	0.286	0.078	0.914	0.858	0.080	0.334	**0.025**	0.491	0.500
JB	0.981	0.785	0.815	0.603	0.758	0.966	0.958	0.636	0.429
HS	0.134	0.313	0.307	0.175	0.357	0.243	0.955	0.462	0.431

Comparison Of Quantity Of GFAP Staining Between NND And SB Brains.

Table 23.
Comparison of quantity of GFAP staining between NND and SB (p values using Mann Whitney U test, significant at <0.05 shown in bold) using computer data.

	NND Median (IQR)	SB Median (IQR)	P Value
Total cases	0.006 (0.003-0.178)	0.004 (0.002-0.006)	0.058
BG	0.018 (0.004-0.019)	0.004 (0.002- 0.006)	**0.015**
HC	0.003 (0.003-0.006)	0.004 (0.003-0.006)	0.411

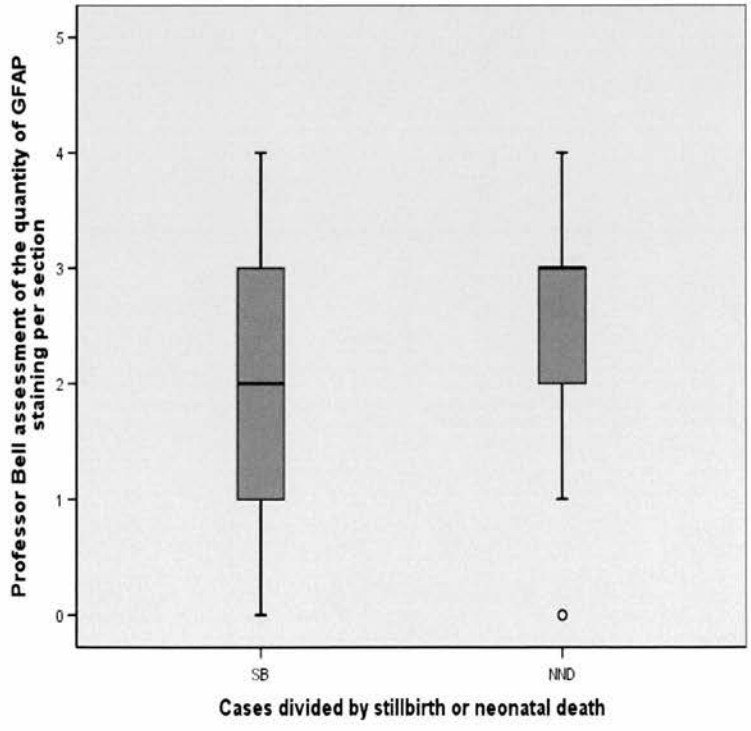
Table 24.
Comparison of quantity of GFAP staining between NND and SB (p values using Mann Whitney U test, significant at <0.05 shown in bold) using JB data.

	NND Median (IQR)	SB Median (IQR)	P Value
Total cases	3 (2-3)	2 (1-3)	0.00
BG	3 (2-3)	2 (1-3)	0.03
HC	2 (2-3)	2 (1-2)	0.08

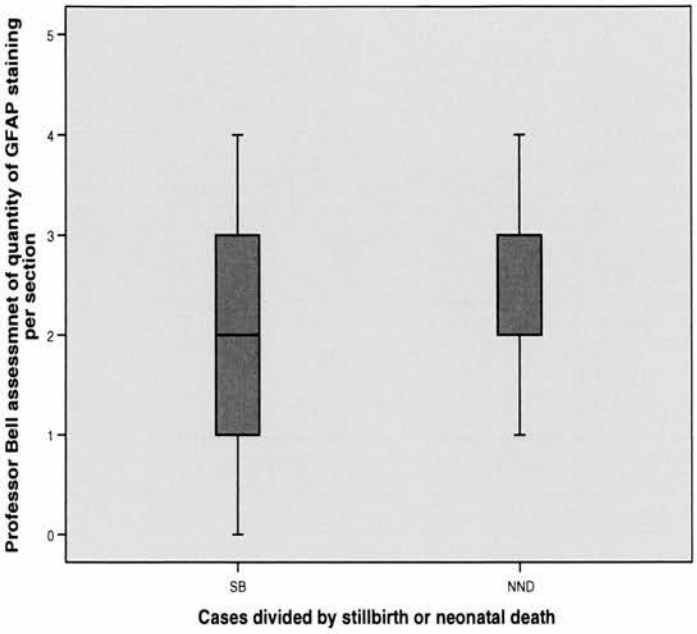
Table 25.
Comparison of quantity of GFAP staining between NND and SB (p values using Mann Whitney U test, significant at <0.05 shown in bold) using HS data.

	NND Median (IQR)	SB Median (IQR)	P Value
Total cases	2 (1.48-2.67)	2.15 (1.69-2.68)	0.64
BG	2 (1.50-2.80)	2.25 (2.00-2.87)	0.65
HC	2 (0.88-2.60)	1.83 (1.66-2.44)	0.77

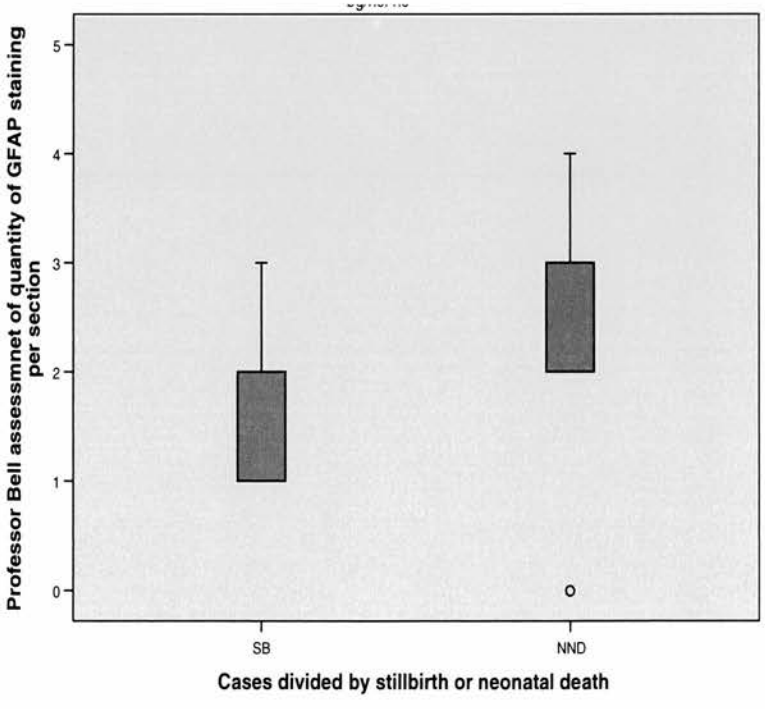
Graph 8.
This boxplot shows the quantity of GFAP staining by visual analysis (JB) comparing NND with SB (all cases).



Graph 9.
This boxplot shows the quantity of GFAP staining in the basal ganglia of SBs and NNDs on visual assessment (JB).



Graph 10.
This boxplot shows the quantity of GFAP staining in the hippocampi of SBs and NNDs on visual assessment (JB).



Comparison Of GFAP Staining Between NND Brains Which Were Documented To Have Some Injury That Predated Delivery And Those That Had Injury That Was Definitely Only Postnatal In Origin.

This shows a comparison of GFAP staining between NND brains which were documented to have some injury that predated delivery and those that had injury that was definitely only postnatal in origin.

Eight out of 23 BG blocks and 6 out of 11 hippocampal blocks were believed to have prenatal damage.

We used the Mann Whitney U test to compare groups.

Table 26.
Comparison of quantity of GFAP staining between cases with no prenatal damage and those with evidence of prenatal damage (p values using Mann Whitney U test) using computer data.

	No prenatal damage Median (IQR)	Prenatal damage Median (IQR)	P Value
Total cases	0.006 (0.003-0.019)	0.006 (0.004-0.013)	0.058
BG	0.012 (0.004-0.020)	0.008 (0.004-0.014)	0.540
HC	0.003 (0.003-0.008)	0.004 (0.003-0.015)	0.119

Table 27.
Comparison of quantity of GFAP staining between cases with no prenatal damage and those with evidence of prenatal damage (p values using Mann Whitney U test) using JB data.

	No prenatal damage Median (IQR)	Prenatal damage Median (IQR)	P Value
Total cases	2 (2-3)	3 (2.50-3)	0.97
BG	2 (2-3)	3 (2.25-3)	0.28
HC	2 (2.20-2.50)	3 (1.50-3.50)	0.14

Table 28.
Comparison of quantity of GFAP staining between cases with no prenatal damage and those with evidence of prenatal damage (p values using Mann Whitney U test) using HS data.

	No prenatal damage Median (IQR)	Prenatal damage Median (IQR)	P Value
Total cases	2 (1.36-2.63)	2 (1.70-2.73)	0.66
BG	2 (1.33-2.8)	2.55 (2-2.92)	0.16
HC	2.08 (1.65-2.61)	1.65 (0.79-2.38)	0.46

Discussion

There is much debate regarding expression of Myelin Basic Protein and Glial Fibrillary Acidic Protein, in particular with how their expression is related to the presence of brain injury and how the protein expression varies with gestation.

The two stains (MBP /GFAP) used for this work allowed analysis of two different cell types: oligodendrocytes and astrocytes. The purpose of this was to see if the different types of cells were affected differently. Pre-Oligodendrocytes (pre-OLs) appear to be the main target in diffuse PVL and they are the main cell line present at the time of maximal development of PVL;18-27 weeks[58,58]. At this gestational age pre-OLs make up 90% of the oligodendrocyte lineage that is present[58,58], indeed the death of these cells could be the trigger to reactive astrocytosis. In diffuse PVL there is reduced myelination giving the tissue a certain pallor and reduced staining to MBP. This is related to both loss of oligodendrocytes through necrosis and also damage to surrounding cells, but with relative sparing of other cells more distant from the injury. This may imply that what damages pre-OLs is not the same as what causes the death of axons or other glial cells[185].

In our study we wanted to compare the types of brain injury seen in a cohort of stillbirths when compared to a cohort of neonatal deaths. If a difference was seen we hypothesised that this was related to some extra uterine factor that the stillbirths have not been exposed to e.g. oxygen. We specifically chose to look at the hippocampus and basal ganglia as these areas are commonly affected in prematurity related brain damage. Using brains from stillbirths as the control group has enabled us to ascertain if any postnatal factor has contributed to the brain injury seen.

Myelin Basic Protein

There are two conclusions from this body of work. Firstly, that the expression of MBP is not quantitatively different between a group of stillbirths and a group of neonatal deaths. Secondly, expression of MBP does correlate with gestation within a cohort of neonatal deaths and in a cohort of stillbirths.

Comparison Of Staining Between Stillbirths And Neonatal Deaths

A direct comparison of quantity of staining between stillbirths and neonatal deaths did not show any statistical significance (tables 6-8). This was unexpected, as we had anticipated that brains from neonates who had suffered a neonatal death might show decreased MBP expression as a marker of a greater degree of brain damage as compared with brains of stillborn infants of comparable gestation. This does not confirm our hypothesis that postnatal oxygen may contribute to some of the white matter injury seen in surviving infants.

Despite the absence of a significant difference, there is a suspicion that MBP expression is less in the neonatal death cohort. Although the lack of significance may well be genuine, it may reflect a general delay in myelination seen in these brains as a consequence of the pregnancy not progressing as expected, which has not been demonstrated because of inadequate numbers. Delayed myelination has been described within the American National Collaborative Perinatal Project[189]. The 15%

of newborn infants with the lowest myelination scores were deemed to have delayed myelination. This conclusion was linked to cigarette smoking, low birth weight and gestational age less than 36 weeks. As our groups were matched for weight and gestation, the trend of less MBP expression in the NND that we have shown clearly cannot be attributed to this[189].

Alternatively there could be a postnatal factor that is affecting the MBP expression that is not present in the stillborn cohort. If it is assumed that stillbirth and neonatal death are all part of one continuum of abnormal pregnancy then it could be deduced that the less MBP seen in the neonatal deaths must be specific to the ex-utero environment. Although some data are lacking, very few of either cohort had antenatal or postnatal infection confirmed. As the cohort is small we are unable to quantify the affect that prolonged rupture of the membranes had on these results. We propose that the explanation for this possible difference in MBP expression between the two groups is postnatal oxygen, which shall be discussed later.

The possible different expression of MBP in the neonatal death cohort could be due to a variety of factors. There could genuinely be no effect on myelin basic protein expression or the injury sustained in these infants may affect the oligodendroglia at later stages of development once MBP has been expressed. However, while this may be true for the more mature infants, it is unlikely to be the explanation at earlier stages of gestation, as both cohorts were matched for gestation and included infants from the threshold of viability until term. The problem with trying to make conclusions relating to gestational age is that the majority of brains studied were from more mature infants i.e. those in whom we would be unlikely to see diffuse white matter damage relating to injury to immature oligodendrocytes because at these later gestations the oligodendrocytes are more mature and resistant to injury.

The overwhelming confounding factor though in this comparison has to be that a pregnancy ending in still birth has clearly not progressed normally for some reason. In many cases this is multifactorial and in others it is not identified at all.

Our results were also assessed in relation to evidence of prenatal brain injury. There was no difference in staining between the two groups that were subdivided in to prenatal or no prenatal injury (tables 9-11). One observer HS made a decision regarding presence of prenatal injury. This was done on the basis of comments regarding gliosis and potential timing of injury within the pathology report though, unfortunately, in many post-mortem reports this was not commented on. One observer (JB) reviewed each case and a final decision was made regarding prenatal injury. Significant prenatal injury was ascertained by the presence of established infarcts, previous haemorrhage or extensive mineralisation. Diffuse features like macrophage infiltration and accumulation or the development of prominent astrocytic cell hyperplasia are also thought to take more than three days to establish and are, therefore, likely to be prenatal in origin in those babies who died in the first few days of life[96].

I have been unable to show any significant difference in staining between the group with prenatal damage and those without. This may be a type two error as numbers in

both groups are small especially when divided into anatomical regions: 9 basal ganglia and 5 hippocampi.

Becher et al showed that 35% of stillbirths had evidence of brain injury that predated any form of terminal event, suggesting that in many of these pregnancies there are long standing problems affecting the infants' development. The most significant antenatal factors associated with this injury were related to abnormal placental function; pregnancy induced hypertension and low placental weight[175]. Kumazaki et al performed a study looking at placental pathology and its association with periventricular leukomalacia. They showed that in 41.7% infants with PVL there was evidence of placental damage: thrombosis, infarction or retro placental haematoma. Only 13.7% of the control group without PVL showed any of these changes[197]. Unfortunately, owing to lack of detailed placenta data this analysis cannot be replicated.

Quantity Of MBP Staining And Its Relationship To Gestation

There is much debate regarding MBP expression and its relationship to gestation of the infant. It is completely plausible that expression increases with gestation. We know that the process of myelination has only just commenced at the fourth month of gestation and increases along with an increase in the mature MBP positive oligodendrocyte population. This process gradually progresses through the cerebrum in the third trimester and into early childhood[183].

All neonatal deaths in our study had occurred within one week of birth. It was felt that within this small time frame, when investigating the relationship of MBP expression with gestation there would be little effect on protein expression. As the infants lived for a variable length of time (median 32 hours, IQR 24-72) a decision was made that age for the purposes of the study was the gestation at birth.

MBP expression increased as gestation increased in this entire cohort of both stillborn babies and those experiencing neonatal death ($p=0.000$) (table 5). There were 4 subgroups out of 27 where MBP expression did not appear to increase in line with gestation (HS analysis of stillbirths including when divided in to anatomical areas, and JB analysis of the stillborn hippocampi). The majority of the results from the Spearman correlation were very significant with p values <0.005.

Although the increase in expression in the stillbirth group is in line with increased gestation, this is not the sole contributing variable. In table 5, I have shown the correlation coefficients for all subgroups of the study. The group with the least association between staining and gestation are the basal ganglia of stillbirths assessed by HS where $r^2=0.08$ so only 8% of the variation in staining can be attributed to increase in gestation. The best subgroup is the hippocampi as assessed by computer. This group have an $r^2=0.644$ thus 64% of the variation in staining can be attributed to increase in gestation.

The different areas of the brain have been separated within the analysis. This is because the two areas myelinate at different ages and so the results are not comparable.

Simply looking at the lack of protein expression does not give any information about when the damage occurs in the process of oligodendrocyte development. It could be that the neonatal brains were appropriately myelinated at the time of delivery as the pregnancy was progressing well and then some postnatal factor turned off MBP expression prior to death leading to a failure in the normal progression of myelination. As our infants all died within the first week of life this would be difficult to ascertain. In contrast the stillborn cohort were not myelinating normally in-utero prior to the final fatal insult. The in-utero processes could well have been having an effect on the pre-OLs prior to delivery. Cowan et al concluded that within the HIE cohort the majority of injury seen on MRI scans occurred around the time of delivery with very little damage predating that time. It is generally considered that for many infants with HIE the sentinel event is possibly pre-terminal and as such if the infant has not been delivered in a timely fashion they may have been a still birth[177]. This MRI evidence suggesting there is very little prenatal injury in these infants is the exact opposite of what Becher et al propose with their post-mortem study. This discussion may be resolved with improved post-mortem MRI techniques with greater resolution of images to enable the pathology to be related to the MRI data acquired.

Oxidative stress is one mechanism of injury that affects these cells. We have hypothesised that it would be present in our neonatal death specimens but not the stillbirths and relates directly to the relative hyperoxia experienced by the infants. Animal and human studies have confirmed that oligodendrocytes are very sensitive to hyperoxic injury[107,106]. The animal studies often use extremes of hyperoxia; FiO2 80% alternating with room air[106]. This is much more extreme than usual clinical practice within the NNU. Research from Edinburgh has shown that there is a reduction in MBP expression in rat brains, which have been exposed to a clinically relevant oxygen profile i.e. inspired oxygen concentration mean of 8kPa with fluctuations for fourteen days[107]. Our neonatal cohort of infants had a very unstable clinical course during their lives. All required significant ventilation and postnatal oxygen with many remaining acidotic and never achieving a normal pH. It is possible that some were subjected to periods of hyperoxia but also, during this critical period of postnatal adaptation, that their antioxidant defence mechanisms were inadequate for even the oxygen levels thought appropriate for normal term infants.

GFAP

We conclude that the brains of neonatal deaths demonstrate significantly more reactive astrocytosis than those of stillbirths. This is particularly true in the basal ganglia. We also conclude that GFAP expression does not correlate with gestation.

Comparison Of Staining Between Stillbirths And Neonatal Deaths

We have demonstrated that there is an increased amount of reactive astrocytosis seen in the white matter of the basal ganglia in infants who had experienced a neonatal death when compared to a similar cohort of stillbirths (tables 14-16).

This effect may be greatest in the basal ganglia because this area is believed to have higher metabolic demands than other regions of the brain. This is one of the regions

that is selectively damaged in response to hypoxia around the time of delivery. At term gestation this central area within the internal capsule is undergoing myelination and as such is exquisitely sensitive to any biochemical or oxidative changes within the brain. Our results also suggest that it may also be sensitive to hyperoxia.

Reactive astrocytosis occurs between 2-4 days after injury. This has been demonstrated in experiments where astrocytes have been labelled by 3H Thymidine at different time points. There is an increase in labelled cells for the first 4 days following injury but there is no difference after this time point[196]. Many of our infants did not live long enough to conclusively implicate postnatal factors in the damage seen. To stratify for this the results were analysed by dividing the data in to 2 groups: those with evidence of prenatal damage and those without. The amount of data were small when divided in to brain regions (8 basal ganglia cases and 11 hippocampal cases). We did not demonstrate a significant difference between those experiencing prenatal damage and those whose damage was felt to be only postnatal in origin. This is again likely to be a type two error due to the small sample size. We would have expected there to be a difference in the two groups as we hypothesise that brains damaged prenatally have less capacity to cope with a postnatal insult and thus develop an exaggerated response to injury when compared with those brains deemed to be normal at time of delivery.

Quantity Of GFAP Staining And Its Relationship To Gestation

We did not demonstrate any correlation in the amount of GFAP staining and increasing gestation in any of our subgroups (table 13). The relationship between GFAP staining and gestation is controversial. Initial cellular responses in the white matter are related to the presence of histiocytes as the astrocytes are immature. Eventually an astrocytic response is visible around 6 months of gestation[187]. It would be expected from this developmental process that there might be changes with the response seen with further increases in gestation as seen with MBP.

The gliosis caused by the astrocytes may not be as evident within the neonatal brain as expected. This is because the immature brain has an immature immune system and may not be able to mount an adequate response to the injury. Balasingam et al demonstrated this in a neonatal rodent model of traumatic cerebral injury. In this they took 3 day old mice and gave them a stab wound to the brain. 4 days later the animals were sacrificed and GFAP staining of the injured area was carried out to assess the amount of reactive astrocytosis. They showed that the injury sustained for the longer duration results in a greater amount of GFAP positive astrocytes being present. This increase was also created by administering additional cytokines to the site of injury: γIFN, IL-1α/β, IL-2, IL-6 TNF-α and macrophage colony stimulating factor (m-csf). They conclude that a decent astrocytic response can be elicited in the neonatal rodent brain if there is sufficient stimuli i.e. duration of insult and potential additive factors[198].

Kalman et al investigated the timing of astrocytic response in a neonatal mouse model of traumatic cerebral injury. They injured mice as both embryos (E18-20) and then at various postnatal ages (P0, P4 and P6). The injury involved a " light" stab injury of a thin disposable needle or a "severe" injury using a wider bore needle

under suction. The animals were then also sacrificed at differing time points so that there could be an analysis of the amount of reactive astrocytosis present and the timing of its development in relation to the time and type of injury. Their conclusion was that the more mature mice developed a larger response to each injury when compared to their less mature counterparts. The more mature rodents also demonstrated this response in a shorter time period than the less mature group[199].

This developmental aspect of the astrocytic reaction has also been seen in the human brain. The NCPP showed that the presence of reactive astrocytes increased from 9% in preterm infants to 59% in term infants[189]. In the clinical situation we are examining in this study it may be worth repeating the experiment on brains of infants who have survived even longer to see if the prolongation of the hyperoxic insult increases the amount of reactive astrocytosis present. This idea makes sense when it is accepted that the presence of reactive astrocytosis takes up to three days to develop. Therefore it is reasonable to conclude that some of our infants may not have lived long enough to develop the full astrocytic reaction to the injury in spite of being subjected to the hyperoxia. This may also result in an underestimation of our results.

The Role Of Oxygen In The Pathology Seen

Extremes of hyperoxia are known to cause death of rodent preoligodendrocytes in vitro[106]. Pre-oligodendrocytes in cell culture were exposed to 6,12 or 24 hours of 80% oxygen. This hyperoxia exposure was repeated in Wistar rats at differing ages (P3, P6 and P10) all of whom were killed at P11 and sections were stained for MBP. The hyperoxia caused cell death in preoligodendrocytes but not in mature OLs. In the rats the results were significant at P3 and P6 but not at P10. The effect was directly related to the duration of exposure to oxygen and was due to the presence of free radicals as there was an increase in the amount of superoxide and other reactive oxygen species present after the oxygen exposure. This in turn led to an increase in the caspase dependent apoptotic cell death pathway in the immature oligodendrocytes[106].

Astrocytes are exquisitely sensitive to oxidative damage. At times of even borderline hyperoxia the premature infants anti-oxidant capacity is overwhelmed. It has been demonstrated in mice astrocyte cell cultures that there is an increase in astrocyte cell death when they were exposed to hydrogen peroxide. To confirm the cell death quantification was made of the amount of lactate dehydrogenase present. An antioxidant was added to the culture, which reduced the amount of cell death and this confirmed that the cell death seen was related to oxidative damage and not some other process. This effect is exaggerated when free iron is added to the culture. This is highly relevant as there is much free iron within the developing brain[200].

At times of ischemia, there is a reduction in the amount of high energy phosphates within the brain resulting in an increase in concentration of hypoxanthine. At the time of reperfusion, this is then broken down by xanthine oxidase in the presence of oxygen into uric acid and the free radical superoxide[165]. During ischemia there is an increase in intracellular calcium. This activates nitric oxide synthase and results in the formation of nitric oxide. Superoxide binds with nitric oxide to form peroxynitrite the most potent of all free radicals, which causes significant white

matter damage (also to neurons). Peroxynitrite causes damage by crossing lipid membranes, damaging DNA and inhibits its repair, which leads to protein damage. It causes disruption in the mitochondria by reducing ATP synthesis and induces cell death. It can also activate caspases that trigger apoptosis, which leads to an increase in cell death and therefore increased white matter injury.

Limitations

Post mortem specimens from stillbirths are not the perfect control group for a group of neonatal deaths but there are no alternatives. SIDS brains have been used before. However, until the pathology of SIDS is better understood, they present a range of potential confounders to any study. At least stillbirths should be similar to neonatal deaths and are felt to be one end of the same spectrum of disease. Stillbirths were the ideal control group for our study as we wanted to assess the effect postnatal oxygen exposure had on the infants. Clearly stillborn infants have never been exposed to potentially harmful postnatal oxygen.

The basal ganglia and hippocampus were chosen as they were felt to be the areas most likely to demonstrate damage that could be attributed to postnatal oxygen administration. It would be interesting to assess the protein expression in other areas of the brain as well.

Our sample size was reasonable but when divided into differing ages there were only 1 or 2 samples per gestational week. The NNDs were well matched with the stillborn cohort for sex and gestation. The discrepancy in case number is due to a variety of factors. In some cases blocks from one or other anatomical areas were unavailable or unidentifiable. In addition, some sections that were available were unfortunately damaged during processing.

There is no perfect method for quantifying histological staining. We used both computer and manual assessment of the sections for our analysis. With this data we have been able to make an assessment of the accuracy of the computer software against an expert's (JB) assessment of the same slides. The correlation of scores between human and computer analysis was very comparable when assessing GFAP and MBP staining. The GFAP analysis being exceptionally accurate (weighted kappa 0.63 and 0.66) which further validates the use of computer technology in histological analysis. The computer randomly selected areas for study. This was the ideal we were aiming for with the manual analysis but we will have been instinctively drawn to assessing certain areas of the sections compared to others. The computer may be more accurate as a threshold of intensity of staining is picked and this is standardised for all sections analysed whereas the human eye may not be as precise when assessing quantity of staining in this manner. The slides were assessed in a blinded fashion but to an experienced observer such as JB there would be other clues within the section that would indicate the timing of injury and general condition of the brain, which may have affected the assessment of the staining pattern.

Conclusion

We conclude that the pattern of MBP expression is not different between a group of stillbirths and neonatal deaths but the quantity of staining present is related to

gestation. GFAP expression is increased in the basal ganglia of infants subjected to a neonatal death rather than stillbirth. The expression of GFAP does not vary with gestation.

We hypothesise that the effect seen is related to some postnatal factor that is not present in the stillborn population and we are implicating oxygen in this. A premature infant develops normally in the relatively hypoxic uterine environment. At the time of premature birth they are placed in a relatively hyperoxic environment, even without additional oxygen. The infant is unable to safely handle the additional oxygen; this affects their neurodevelopment and is a factor contributing to the diffuse white matter damage seen in this group of children. We are aware a major confounder to this is that the majority of our brains studied were from more mature infants where the injury is less likely to be related to immature oligodendrocytes.

CONCLUSION

Oxygen is essential for life and is used on a daily basis within the neonatal unit. It has been known for over 50 years that excess is damaging to premature infants causing excessive cases of blindness. There is gathering evidence that it may also be harmful to the lungs. As neonatal care improves we are gaining an ever-increasing population of survivors and this surviving population is not without significant neurodevelopmental morbidity. One of the next great challenges within neonatology is to identify modifiable factors within the infants' clinical course that impact on their neurodevelopmental outcome. The aim of this thesis was to see if oxygen could be implicated in this neurological damage. The results of the different studies may not by singularly significant but certainly suggest that oxygen may have a significant role to play in the pathology of premature brain injury.

The animal work was set up to assess the vascularity of the rodent brain when subjected to variable oxygen as opposed to room air. The hypothesis was that if variable oxygen can lead to ROP then the same pathological process could be continuing within the brain as well.

Histological examination of sections of the brain in the areas of cortex, hippocampus and internal capsule were assessed for both capillary density and capillary diameter. Both of these parameters are affected in models of hypoxic injury. The results showed that, although not statistically significant, there was a trend for there to be more capillaries in the brains of rodents reared in variable oxygen. These capillaries also have a trend to being of wider diameter. In a model of mild physiological hyperoxia it seemed counterintuitive for the vessels to be responding as they would in a hypoxic environment. It was noted that part of the pathology of ROP is in fact disordered vessel overgrowth, with an increase in capillary numbers even if they do not effectively cover the retinal area. This is thought to be in part to the retina experiencing periods of relative hypoxia due to hyperoxia leading to a reduction in angiogenesis because of a down-regulation of HIF1α and consequent reduction in VEGF. This hypoxia causes a resultant increase in HIF1α and VEGF leading to an increase in angiogenesis and so the cycle starts again. The vasculature as a result is disordered and more permeable leading to the pathology seen in ROP.

It is with this in mind that we were delighted with the results from Magnetic Resonance Angiography that showed that the major vessels in a rodent brain are smaller volume when the pup is reared in variable oxygen when compared to room air. This possible vasoconstriction proximally within the vasculature could explain the hypoxic appearances we saw more distally.

We could not demonstrate any difference in vessel tortuosity as seen in the preterm infants scanned at term. This was not surprising as the rodent brain is a much less complicated structure than the human and so unlikely to have an overly complex vasculature. The rodent brain is also much smaller than the human brain and so the detail of the vasculature could not be assessed as accurately.

The main limitation of the study is the lack of pups assessed, due to practical and timing issues. The main advantage of the study is the lack of confounding factors. The only difference between the two groups was the use of variable oxygen. The groups were identical in every other way. This is not possible within the confines of a clinical study.

The human study was set up to try and establish if there was any difference in pathology seen between infants who experienced stillbirth and those who experienced a neonatal death. This was an extension of the much larger Scottish Perinatal Neuropathology Study. The hypothesis was that any difference in pathology seen could be attributed to the use of oxygen postnatally. Assuming that stillbirth is part of the same spectrum of disease as neonatal death the babies would be similar in many respects apart from their exposure to oxygen prior to death.

There were a large number of cases used in this part of the study, although once divided into subgroups they were unlikely to reveal any significant results. The study concluded that there is an increased astrocytic response to injury in the cases that had experienced a neonatal death. This is especially true in the basal ganglia. There appeared to be no difference in the effect this had on myelination assessed by use of the antibody to myelin basic protein. When the cases were divided into subgroups where the injury pattern implied a definite prenatal cause as opposed to those where the injury was deemed to be postnatal there was no difference in the damage seen.

The study did show that expression of myelin basic protein increased along with an increase in gestational age of the infant. This effect is not seen with glial fibrillary acidic protein.

There are many clinical factors that will have confounded this study. To minimise this the cases were matched for sex, gestation and birth weight as closely as possible. Unfortunately as the majority of brains used were from more mature gestations it is difficult to make any sensible conclusions that reflect our hypothesis. The mature oligodendrocyte seen in latter gestations is not as sensitive to oxygen related damage as its more immature predecessor.

Although on their own none of the studies are statistically significant we hope that this work will add to the body of evidence that is gradually accumulating implicating postnatal oxygen use with some of the brain injury seen in survivors of preterm birth. This experimental evidence combined with clinical studies, may alter clinical practice and see a further restriction in the amount of oxygen administered within the NNU.

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APPENDICES

Appendix 1.

Rodent Brain And Eye Dissection

Anaesthetic
0.3ml of Vetalor – Xylazine hydrochloride
0.6ml of Rompon – ketamine use 0.2ml per 30 g
0.9ml of PBS

Perfuse with
2-10ml PBS
2-5ml of 4% PFA

Dissection of the brain
The brains should be cut as fresh as possible as they begin to soften quickly. Keep them on cold blocks from -70˚C freezer
Place individual vials for separate parts of brain on cold blocks to cool for 15 mins then start dissecting the brain

Have brain upside down – remove any remaining spine
Remove olfactory bulb from front
Remove hypothalamus – round bit on top as you look at it
Peel brain stem off midbrain and cerebellum
Remove cerebellum – straight cut
Remove midbrain – inverted V shape from centre of matter left
Hippocampus – oblong structure bilaterally, dissect and liftout
Striatum – gently remove this fleshy structure from the overlying cortex
Cortex – this is all that is left and can be easily rolled up and placed in vial

Place in -70˚C freezer until needed for western blotting

Cutting the brain
Leave brains in PFA from day of sacrifice for 4-5 days
Then cryoprotect in 30% sucrose until they sink, around 24-48hours
Replace sucrose if necessary

Take to freezing plate on microtome
Place fixative on plate
Place filter paper on top
Once going opaque – it is beginning to freeze
Add further fixative to keep the brain vertical
Cover with silver foil
Let temp drop to -44 degrees – which will take around 30mins
Once solid re-warm to between -32 - -28 degrees, allow temp to equilibrate over next 10-15 mins, prepare 12 well plates for sections with PBS in each well.

Appendix 2.

CD 31 Immunohistochemistry

The sections of rat brain are stored in the fridge in PBS until needed for staining. The required sections are then removed from the fridge and placed in wells of PBS ready for staining. Each brain is stained in a separate well, the specific anatomical areas being separated at the time of mounting the sections on the slides. (For manufacturers details of solutions used see appendix....)

The sections should be washed in PBS triton 1% by transferring them gently on a paintbrush from a well of PBS into the triton. This should be done twice to ensure maximal washing. The sections are then placed in a well of hydrogen peroxide 3% for 5 minutes to block any natural occurring peroxidases. The sections are then washed in PBS triton 1% again.

The sections are then placed in blocking swine serum for 30 minutes and then transferred in to the primary antibody mixed with blocking serum – Goat anti-CD31 1:100 and left overnight at room temperature. A well of control sections is simply left in the blocking buffer over night.

The sections are then washed with PBS triton 1% and then placed in the secondary antibody mixed with PBS- Swine anti-goat 1:200 and left for 2 hours. They are washed again in PBS triton 1% and then placed in Strep ABC 1:200 mixed with PBS for 1 hour. From this well the sections are transferred in small numbers to the diaminobenzidine for counterstaining. The sections are then mounted on slides according to their anatomical area for ease of analysis and left to dry over night.

The sections are then rehydrated with PBS and immersed in Thionin for 2-3 minutes. Then placed in 70% alcohol containing a few drops of glacial acetic acid for 2-3 minutes and then dehydrated using 70/90/100% alcohol and finally cover slipped ready for analysis.

Appendix 3.

Rat Data Appendix

	Room air (g)	Oxygen (g)
Weight	5.79	5.53
	5.81	5.4
	5.34	5.34
	5.52	5.22
	5.13	6.28
		7.31
Mean	5.51	5.84
SD	0.29	0.81
P value	0.39	

Average birth weight for litters used in experiments

End weight / brain weight and percentage body weight for pups analysed
P value comparing room air and oxygen reared pups

	End weight (g)	Brain weight (g)	% body weight
Room air	28.6	1.1	0.04
	27.4	1	0.04
	29.25	1.15	0.04
	26.9	1.1	0.04
	29.2	1	0.04
Mean	28.3	1.07	0.04
SD	1.06	0.07	0
Oxygen	28.65	0.95	0.03
	27	1.1	0.04
	23.35	0.95	0.04
	23.6	1.05	0.05
	23.75	1.1	0.04
	26.2	1.05	0.04
	23.3	0.95	0.04
Mean	25.3	1.02	0.04
SD	2.02	0.07	0
P value	0.01	0.26	0.75

Appendix 4.

CD 31 Average Vessel Count Per Rat Pup In Each Of Three Separate Anatomical Areas

Cortex Vessel Count

RA	14.76
RA	7.64
RA	7.11
RA	4.74
RA	9.27
RA	5.16
RA	6.96
RA	7.61
RA	17.97
RA	4.60
RA	0.74
RA	30.00
O2	14.21
O2	28.90
O2	5.68
O2	18.54
O2	17.53
O2	27.14
O2	14.16
O2	2.16
O2	7.14

Hippocampus Vessel Count

RA	57.79
RA	14.05
RA	7.58
RA	12.10
RA	33.33
RA	8.60
RA	18.63
RA	26.24
RA	3.50
RA	57.48
O2	13.50
O2	33.50
O2	8.27
O2	27.08
O2	28.69
O2	29.60
O2	9.70
O2	15.72
O2	33.90

Internal Capsule Vessel Count

RA	42.29
RA	25.59
RA	2.34
RA	3.74
RA	9.65
RA	12.25
RA	3.95
RA	23.59
RA	21.62
RA	1.72
RA	43.63
O2	8.27
O2	14.51
O2	0.12
O2	5.74
O2	12.59
O2	14.16
O2	11.47
O2	3.49
O2	9.38

Appendix 5.

CD 31 Average Vessel Diameters Per Rat Pup In Each Of Three Separate Anatomical Areas

Cortex Vessel Diameters

Room air / oxygen	Diameter microns
RA	2.33
RA	2.95
RA	2.40
RA	4.55
RA	2.28
RA	4.60
RA	3.47
RA	1.79
RA	2.96
RA	2.05
RA	2.98
RA	1.64
O2	3.60
O2	3.06
O2	3.03
O2	3.33
O2	2.38
O2	3.27
O2	3.70
O2	3.49
O2	4.07

Hippocampus Vessel Diameters

Room air / oxygen	Diameter microns
RA	2.19
RA	2.87
RA	2.97
RA	2.69
RA	3.50
RA	2.93
RA	1.63
RA	2.98
RA	2.48
RA	2.17
O2	3.89
O2	2.66
O2	2.85
O2	2.81
O2	2.61
O2	3.26
O2	2.70
O2	2.84
O2	3.72

Internal Capsule Vessel Diameters

Room air / oxygen	Diameter microns
RA	2.49
RA	2.70
RA	2.81
RA	2.54
RA	3.64
RA	3.1
RA	2.89
RA	1.84
RA	2.23
RA	4.09
RA	1.86
O2	3.75
O2	2.77
O2	2.41
O2	3.21
O2	1.93
O2	2.84
O2	3.74
O2	2.62
O2	3.94

Appendix 6.

MRA Data

Tortuosity Distance factor		
	Oxygen	Room air
Right ICA	1.15	1.013
	1.156	1.11
	1.043	1.02
Left ICA	1.13	1.03
	1.143	1.11
	1.043	1.01
Total ICA	1.14	1.0215
	1.15	1.11
	1.04	1.015
Right MCA	1.063	1.043
	1.05	1
	1.03	1.05
Left MCA	1.023	1.05
	1.08	1
	1.036	1.04
Total MCA	1.04	1.045
	1.065	1
	1.03	1.045
Right ACA	1.063	1.06
	1.063	1.053
	1.016	1.063
Left ACA	1.1	1.04
	1.1	1.076
	1.023	1.03
Total ACA	1.0815	1.05
	1.0815	1.063
	1.02	1.04
Basilar	1	1.05
	1	1.06
	1.11	1.04

Volume mm^3		
	Oxygen	Room air
Right ICA	0.44	1.71
	0.65	0.57
	0.55	1.31
Left ICA	0.49	1.78
	0.62	0.79
	0.55	0.81
Total ICA	0.47	1.745
	0.64	0.68
	0.57	1.06
Right MCA	0.25	0.49
	0.4	0.68
	0.59	0.59
Left MCA	0.3	1.13
	0.33	0.51
	0.58	0.71
Total MCA	0.275	0.81
	0.37	0.6
	0.59	0.65
Left ACA	1.27	0.69
	0.52	0.37
	0.57	0.7
Right ACA	0.56	2.65
	0.45	0.52
	0.67	0.4
Total ACA	0.62	1.96
	0.41	0.52
	0.69	0.48
Basilar	0.19	0.22
	0.16	0.98
	0.68	0.78

Appendix 7.

Staining For Myelin Basic Protein (MBP)

The paraffin was removed from the slides using xylene and absolute / 74% and 70% alcohol then transferred into picric acid for 30 minutes. The slides were washed in running water and then placed in a bath of Citric acid pH 6 for 15 minutes on high power in the microwave. The citric acid was made up using 1.57g of solid citric acid mixed with 750mls of distilled water. Once cool, the slides were again washed in water then transferred to a bath of 3% hydrogen peroxide for 10 minutes.

The slides were then further washed in water and then Tris Buffered Saline (TBS) and then left in normal swine serum (NSS) for 10 minutes. The slides were then placed in a sequenza rack and exposed to the primary antibody (MBP 1:1000, (Dako)) made up with NSS and then left for 30minutes. The negative control slides were simply exposed to more NSS.

Following a wash in TBS, the slides were exposed for 30 minutes to the secondary antibody : biotinylated anti-rabbit immunoglobulin (SARBO, Dako) made up to 1:200 dilution in NSS,

Lastly, the slides were washed in TBS and then exposed to Streptavidin biotinylated horseradish peroxidase complex (Strept ABC, Dako) for 30 minutes. They were then washed, treated with DAB and counterstained using haematoxylin.

All sections were stained within the same day to ensure standardisation of solutions and conditions.

Appendix 8.

Staining For Glial Fibrillary Acidic Protein (GFAP)

The paraffin was removed from the sections using xylene and absolute / 74% and 70% alcohol then treated with trypsin for 20 minutes in a 37°C water bath. The trypsin was made using 0.75g trypsin, 0.75 g of calcium chloride and 750 mls of TBS made to a pH of 7.6. Again the slides were placed in a sequenza rack. The slides were the treated with NSS for 10 minutes, then exposed for 30 minutes to the primary antibody (GFAP 1:2000(Dako)) made up with NSS.

The slides were then washed in TBS and exposed to SARBO at 1:200 for 30 minutes, and then further washed in TBS and exposed to Strept ABC for 30 minutes. The slides were then exposed to DAB and counterstained using haematoxylin.

All sections were stained within the same day to ensure standardisation of solutions and conditions.

Appendix 9.

MBP Dataset

MBP data							
Case no	NND / SB	Prenatal damage	Gest	BG /HC	Computer	HS score	JB score
40	nnd	yes	38	hc	0	0	0
234	nnd	no	40	bg	841.9858	0	1
247	nnd	yes	42	hc	94181.2	0	2
263	nnd	no	31	bg	149644	3	3
263	nnd	no	31	hc	52819.33	0	2
1035	nnd	no	36	bg	52627.79	4	4
1097	nnd	no	36	hc	0	0	0
1230	nnd	yes	40	bg	19695.56	1	1
2070	nnd	no	40	bg	26922.33	2	2
2086	nnd	no	40	bg	316194.2	4	3
2156	nnd	no	35	bg	121374.1	3	3
2156	nnd	no	35	hc	28048.09	0	2
2170	nnd	yes	25	bg	93709.4	5	3
2217	nnd	yes	25	bg	11762.9	3	2
3121	nnd	no	28	bg	149378.1	3	3
3135	nnd	yes	29	bg	0	0	0
96067	sb	no	26	bg	380407.5	5	4
96151	sb	no	24	bg	0	0	2
96168	nnd	yes	23	bg	131444.2	3	3
96168	nnd	yes	23	hc	73758.88	2	1
96170	sb	no	34	bg	77551.53	4	3
96170	sb	no	34	hc	0	0	0
96177	nnd	no	24	bg	21472.05	3	2
96179	nnd	yes	25	bg	0	0	0
96179	nnd	yes	25	hc	0	0	0
96181	sb	no	28	bg	18343.03	2	1
96184	sb	no	41	bg	49799.2	3	2
96184	sb	no	41	hc	97743.91	3	2
96234	nnd	yes	36	bg	241724.9	5	3
96234	nnd	yes	36	hc	0	0	0
96235	sb	no	38	bg	29638.55	3	2
96239	sb	no	32	bg	11953.03	0	1
96239	sb	no	32	hc	1803.508	0	0
96286	sb	no	31	bg	45509.67	2	2
96287	nnd	yes	39	bg	241113.4	3	2
96386	nnd	yes	25	bg	227126.2	4	4
96441	sb	no	25	bg	30812.74	3	1
97041	sb	no	38	bg	62937.68	3	3
97041	sb	no	38	hc	45977.91	0	2
97128	sb	no	36	bg	0	0	0
97128	sb	no	36	hc	83224.03	0	0
97178	sb	no	36	bg	207.1013	0	1

97179	sb	no	39	bg	113155.7	4	3
97180	sb	no	36	hc	4194.793	0	1
97193	nnd	no	28	bg	0	0	0
97309	sb	no	40	bg	85271.15	3	3
97319	sb	no	33	bg	6671.379	0	1
97319	sb	no	33	hc	0	0	1
97321	nnd	no	31	bg	9972.835	2	2
97321	nnd	no	31	hc	130150.7	1	3
97329	nnd	no	34	bg	198997.5	4	3
97329	nnd	no	34	hc	84934.48	0	3
98016	sb	no	25	hc	0	0	1
98017	sb	no	25	hc	0	0	0
98022	sb	no	40	bg	22562.16	3	3
98022	sb	no	40	hc	1010.326	0	0
98048	nnd	no	34	hc	40850.24	1	2
98057	sb	no	27	bg	0	0	0
98057	sb	no	27	hc	2339.368	0	1
98093	sb	no	40	bg	76830.99	4	3
99090	sb	no	36	bg	1414503	3	3
99092	sb	no	40	bg	476003.8	3	3
99092	sb	no	40	hc	744421.7	0	3
99096	sb	no	42	bg	650024.1	2	3
99096	sb	no	42	hc	146061.2	0	3
99370	nnd	no	36	bg	0	0	0

Appendix 10.

GFAP Dataset

GFAP data								
Case no.	NND / SB	Predamage	gest age	Sex	Region	HS ave	JB ave	Computer ave
40	NND	yes	33	f	hc	1	1	0.0038
85	NND	no	28	f	bg	2	2	0.0163
120	NND	no	36	f	bg	2	4	0.0264
234	NND	no	34	f	bg	2	2	0.0059
247	NND	yes	38	f	bg	3	3	0.0200
247	NND	yes	38	f	hc	2	3	0.0214
263	NND	no	36	m	bg	3	3	0.0210
263	NND	no	36	m	hc	3	2	0.0215
1035	NND	no	38	f	bg	1	2	0.0131
1097	NND	no	26	f	bg	2	3	0.0200
1230	NND	yes	27	f	bg	2	2	0.0132
2070	NND	no	34	m	bg	1	2	0.0028
2086	NND	no	40	f	bg	3	2	0.0194
2156	NND	no	39	m	bg	2	3	0.0122
2156	NND	no	39	m	hc	3	2	0.0038
2168	NND	no	31	m	bg	3	4	0.0864
2170	NND	yes	40	m	bg	2	3	0.0060
2217	NND	yes	31	f	bg	2	3	0.0041
3121	NND	no	38	f	bg	3	3	0.0064
3135	NND	yes	29	m	bg	3	4	0.0108
96067	SB	no	26	f	bg	2	2	0.0013
96151	SB	no	24	f	bg	1	1	0.0040
96168	NND	yes	40	f	bg	2	2	0.0034
96168	NND	yes	40	f	hc	2	3	0.0034
96170	SB	no	34	f	bg	1	1	0.0000
96177	NND	no	40	m	bg	2	2	0.0038
96177	NND	no	40	m	hc	1	2	0.0014
96179	NND	yes	25	f	hc	1	3	0.0034
96184	SB	no	41	m	bg	3	2	0.0066
96184	SB	no	41	m	hc	2	2	0.0075
96234	NND	yes	35	f	bg	3	2	0.0142
96235	SB	no	38	f	bg	3	1	0.0050
96239	SB	no	32	m	bg	2	1	0.0047
96239	SB	no	32	m	hc	1	1	0.0043
96286	SB	no	31	f	hc	2	2	0.0049
96287	NND	yes	36	m	bg	3	3	0.0037
96287	NND	yes	36	m	hc	2	3	0.0056
96441	SB	no	25	m	bg	3	2	0.0034
97041	SB	no	38	f	bg	3	1	0.0058
97128	SB	no	36	f	bg	2	3	0.0038
97178	SB	no	36	m	bg	2	3	0.0076
97178	SB	no	36	m	hc	2	1	0.0025

97179	SB	no	39	m	bg	2	3	0.0031
97180	SB	no	36	f	hc	2	2	0.0010
97193	NND	no	25	m	bg	1	2	0.0014
97309	SB	no	40	m	bg	3	3	0.0031
97319	SB	no	33	f	bg	2	2	0.0277
97319	SB	no	33	f	hc	2	3	0.0071
97321	NND	no	40	f	bg	3	3	0.0033
97321	NND	no	40	f	hc	2	3	0.0030
97329	NND	no	40	m	hc	2	2	0.0085
98017	SB	no	25	f	hc	2	3	0.0040
98022	SB	no	40	f	bg	2	2	0.0013
98022	SB	no	40	f	hc	2	2	0.0042
98048	NND	no	40	f	bg	3	3	0.0173
98048	NND	no	40	f	hc	3	2	0.0029
98057	SB	no	27	f	bg	2	2	0.0017
98057	SB	no	27	f	hc	3	1	0.0061
98093	SB	no	40	m	bg	3	3	0.0066
99090	SB	no	36	m	bg	2	2	0.0046
99092	SB	no	40	f	hc	2	1	0.0034
99096	SB	no	42	m	bg	2	1	0.0024
99096	SB	no	42	m	hc	3	1	0.0009

Appendix 11.

Oxygen variability causes a reduction in cerebral vessel volume in a rodent model of prematurity.

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Jennifer Brown Research Fund, Edinburgh University, UK

Introduction

The causes of long-term neurological morbidity seen in survivors of premature birth are still largely unknown but studies have revealed that small fluctuations in oxygen tension can cause damage to the cerebral white matter(1). These fluctuations are also part of the explanation for retinopathy of prematurity(2). There is evidence that cerebral blood vessels of premature survivors are different from those of infants born at term in respect of their tortuosity(3). We wished to see if fluctuating oxygen at clinically relevant levels (4) had any effect on the volume of cerebral blood vessels in a rodent model of prematurity.

Method

We compared the cerebral vasculature of 6 new born rats reared for 14 days in either room air or fluctuating oxygen set around a mean of 10kPa. We used Time of Flight Magnetic Resonance Angiography (TOF - MRA) in a 7 tesla small bore scanner. Diet and litter size were standardized.

The rats were sedated using isoflurane for the duration of the scan. Each scan took 15 minutes and the pups had their temperatures and respiratory rates monitored throughout. As this was a pilot study a range of scan parameters were used to obtain the best images but contrast agents were not used. We compared the volume of 4 major arteries in the brain: anterior cerebral artery, middle cerebral artery, internal carotid artery and basilar artery. Vessels were identified within each slice and were reconstructed using tree analysis software. From this the 4 major vessels were identified and the volume of each equal length segment was calculated. For this we used Image J and Analyze 8.1 software.

Results

We found that rats exposed to fluctuating oxygen had significantly reduced total blood vessel volume when compared to the room air control group, p=0.02 . When vessels were compared individually there was a trend towards a similar finding but the results were not statistically significant, probably owing to the small numbers of the pilot study.

Conclusion

It is known that small fluctuations in oxygen tension can cause harm. Where and when most damage occurs needs further exploration. Our data shows that variable oxygen has an effect on the volume of cerebral vasculature. This effect on the vasculature may be the same as that seen in ROP but the mechanism is unclear. It could be an effect of free radical damage as it is known that there is a fine balance between the presence of nitric oxide producing endothelial relaxation and superoxide causing constriction(5). In our model the minimal excess of oxygen may be contributing to this effect. How long lasting this effect is could be established by longitudinal MRI studies.

By combining further animal work with human studies, it may be possible to build up a clearer picture of how oxygen affects vessel development in the brain and whether this contributes in addition to long term neurodevelopmental deficits.

Figure 1. Transverse slice through a rat head. The bright spots represent blood vessels within the head.

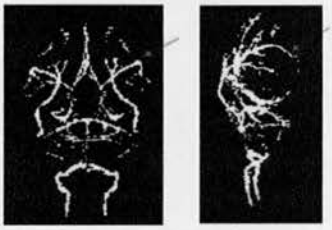
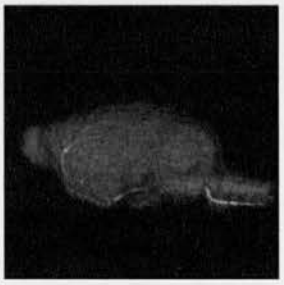


Figure 2. Reconstructed images of the vascular tree in 2 differing planes. The vessel highlighted by the arrow is the middle cerebral artery.



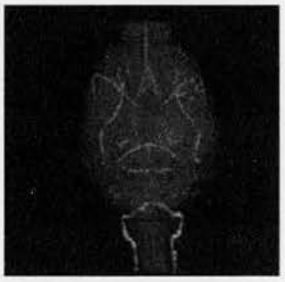


Figure 3. Volume rendered images of the rat brain with vessels shown in red.

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