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Cerebrovascular adaptations to chronic hypoxia in the growth restricted lamb

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ABSTRACT

Chronic moderate hypoxia induces angiogenic adaptation in the brain, reflecting a modulatory role for oxygen in determining cerebrovascular development. Chronic intrauterine fetal hypoxia, such as occurs in intrauterine growth restriction (IUGR) is likely to lead to a reduction in oxygen delivery to the brain and long-term neurological abnormalities. Thus we investigated whether vascular remodeling and vascular abnormalities were evident in the brain of IUGR newborn lambs that were chronically hypoxic in utero. Single uterine artery ligation (SUAL) surgery was performed in fetuses at ~105 days gestation (term ~145 days) to induce placental insufficiency and IUGR. Ewes delivered naturally at term and lambs were euthanased 24 h later. IUGR brains ($n = 9$) demonstrated a significant reduction in positive staining for the number of blood vessels (laminin immunohistochemistry) compared with control ($n = 8$): from 1650 ± 284 to 416 ± 47 cells/mm^2 in subcortical white matter (SCWM) 1793 ± 298 to 385 ± 20 cells/mm^2 in periventricular white matter (PVWM), and 1717 ± 161 to 405 ± 84 cells/mm^2 in the subventricular zone (SVZ). The decrease in vascular density was associated with a significant decrease in VEGF immunoreactivity. The percentage of blood vessels exhibiting endothelial cell proliferation (Ki67 positive) varied regionally between 14 to 22% in white matter of control lambs, while only 1–3% of blood vessels in IUGR brains showed proliferation. A 66% reduction in pericyte coverage (α-SMA and desmin) of blood vessels was observed in SCWM, 71% in PVWM, and 73% in SVZ of IUGR lambs, compared to controls. A reduction in peri-vascular astrocytes (GFAP and laminin) was also observed throughout the white matter of IUGR lambs, and extravasation of albumin into the brain parenchyma was present, indicative of increased permeability of the blood brain barrier. Chronic hypoxia associated with IUGR results in a reduction in vascular density in the white matter of IUGR newborn brains. Vascular pericyte coverage and peri-vascular astrocytes, both of which are essential for stabilisation of blood vessels and the maintenance of vascular permeability, were also decreased in the white matter of IUGR lambs. In turn, these vascular changes could lead to inadequate oxygen supply and contribute to under-perfusion and increased vulnerability of white matter in IUGR infants.

1. Introduction

Intrauterine growth restriction (IUGR) refers to a condition in which a fetus is unable to achieve its genetically determined growth potential. It is most commonly caused by placental insufficiency. IUGR is strongly associated with perinatal brain injury, and may account for up to 50% of neurodevelopmental handicaps and 20% of major neurological sequalae in newborns (Hawdon et al., 1990). The most devastating and permanent injuries that occur in the developing fetal brain are germinal matrix and intraventricular hemorrhage (GMH & IVH) (McIntire and Leveno, 2008), which underlie long-term neurodevelopmental impairments and cerebral palsy. It is generally considered that the incidence and severity of hemorrhagic lesions increases with decreasing gestational age (Papile et al., 1978; Ment et al., 1984; Larroque et al., 2003). Two recent population-based studies have reported that IVH is in fact low at 28 weeks gestation in preterm IUGR infants compared with appropriately-grown preterm infants at the same gestational age,

Abbreviations: IUGR, intrauterine growth restriction; SCWM, subcortical white matter; PVWM, periventricular white matter; SVZ, subventricular zone; VEGF, vascular endothelial growth factor; IVH, intraventricular haemorrhage; GMH, germinal matrix haemorrhage; SUAL, single uterine artery ligation; HIF, hypoxia-inducible factor; BBB, blood brain barrier; alpha-SMA, alpha smooth muscle actin; CBF, cerebral blood flow.

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but that infants born 34–37 weeks gestation show an increased frequency of IVH in IUGR weeks compared with preterm deliveries at the same gestational ages (Ortigosa Rocha et al., 2010), confirming that IUGR is indeed a risk factor for IVH.

Normal brain development and function is dependent on an adequate supply of oxygen. Fetal cerebral vascular beds are one of the most sensitive to poor substrate delivery induced by pathological conditions during intrauterine life. Growth restriction due to placental insufficiency is associated with chronic hypoxia, which, in turn, triggers a redistribution of cardiac output to favor the brain; an adaptive response known as brain sparing (McMillen et al., 2001). Although the fetus is able to adapt to hypoxia by increasing cerebral perfusion, this beneficial blood flow redistribution does not ensure normal brain growth over a prolonged period. The initial increase in cerebral perfusion observed in IUGR fetuses is followed by a pronounced fall in perfusion with progressive fetal deterioration (Hernandez-Andrade et al., 2008). When hypoxia is chronic, as in placental insufficiency, fetal deterioration is characterised by disappearance of physiological cerebral vascular variability (vasoconstriction and vasodilatation) followed by an increase in cerebral vascular resistance, which, in turn, exacerbates brain injury (Salihagic-Kadic et al., 2006). Alterations in cerebral blood flow (CBF) haemodynamics, assessed using Doppler ultrasound, are a hallmark of IUGR (Hernandez-Andrade et al., 2008), and brain sparing is linked to increased risk of abnormal neurodevelopment (Hernandez-Andrade et al., 2012). However, while fetoplacental Doppler studies are useful in both assessing fetal status and guiding timing of delivery, Doppler assessment of middle cerebral artery pulsatility does not measure true changes in cerebral perfusion and, of course, does not assess blood flow to all anatomic areas of the fetal brain. This particularly applies to deeper regions of the brain, such as the white matter. A complex set of neuropathologies are observed in IUGR infants and white matter injury is a significant component in human (Ramenghi et al., 2011; Padilla et al., 2014) and experimental animal IUGR (Olivier et al., 2007; Kelleher et al., 2011; Eixarch et al., 2012; Miller et al., 2014).

Normal brain development requires appropriate networks of blood vessels to supply oxygen and nutrients. The neurovascular unit comprises vascular endothelial cells, ensheathed by a basal lamina membrane, in close association with surrounding astrocytes and pericytes (Hawkins and Davis, 2005). The neurovascular unit is a dynamic and integrated complex of cells and structural components working together to maintain brain homeostasis, regulate blood flow, to maintain integrity of the blood-brain-barrier, and to provide growth factors for normal development and cell function. The present study was undertaken to investigate the adaptive response of the neurovascular unit during development to chronic hypoxia caused by placental insufficiency. We utilized an ovine model of surgically-induced placental insufficiency at approximately 0.7 gestation, and studied components of the cerebrovascular unit within the white matter of the brain in IUGR and control lambs at 24 h after birth. We have previously detailed white matter injury in IUGR lambs, observed as significant hypomyelination and axonal injury (Miller et al., 2014). In this study we hypothesised that vascular development would be adversely affected within the white matter of IUGR lambs.

2. Materials and methods

2.1. Animal surgery and maintenance

The surgical and experimental procedures undertaken in this project were approved by the Monash Medical Centre (A) Animal Ethics Committee, using guidelines established by the National Health and Medical Research Council of Australia. Surgery was performed on 17 Border-Leicester cross Merino pregnant ewes carrying a single fetus at 105 to 110 days gestational age. Prior to the induction of anaesthesia, all ewes received 1 g ampicillin (Austrapen, Lennon Healthcare, St Leonards, NSW, Australia) and 500 mg engemycin (Coopers, Bendigo East, Victoria, Australia) intravenously (i.v.). Anesthesia was induced with i.v. 20 mg/kg sodium thiopentone (Pentothal; Bomac Laboratories Ltd., New Zealand) and maintained with 2% isoflurane (Isoflo, Abott Pty. Ltd., Australia) in oxygen and nitrous oxide (70:30). Under aseptic conditions, the uterus was exposed and a catheter (inner diameter 0.8 mm, outer diameter 1.5 mm, Dural Plastics, Australia) containing 0.9% saline and heparin (25,000 IU/L; Pfizer Australia, West Ryde, NSW, Australia) was inserted into the femoral artery. Single umbilical artery ligation (SUAL) was performed in 9 fetuses by placing two silk ligatures tightly around one of the umbilical arteries and the umbilical cord sheath was then repaired. In 8 control fetuses the umbilical cord was handled but the artery was not ligated. The fetus was returned to the uterus and the uterine and abdominal incisions were repaired. A catheter was inserted into the maternal jugular vein for antibiotic administration.

For 3 consecutive days after surgery, antibiotics were administered to the fetus (ampicillin, 500 mg) and the ewe (engemycin 5 mL). A fetal blood sample (2 mL) was taken every day for 3 days after surgery and thereafter at 115, 120, 125, 130, 135 and 140 days gestation for assessment of fetal wellbeing via arterial blood gases and pH, glucose and lactate (ABL 700 blood gas analyzer; Radiometer, Copenhagen, Denmark). At fetal gestational age 138 days, the ewe was placed under video surveillance and monitored so that the day of labor could be determined (Miller et al., 2014). An experimenter was present at the time of delivery to ensure that the fetal catheter was cut and that the lamb was delivered and cared-for by the ewe.

2.2. Preparation of the tissues

Lambs were euthanazed 24 h after birth by intravenous injection of pentobarbitone sodium (Lethabarb;Virbac Pty., Ltd., Peakhurst, NSW, Australia). The brain was removed, weighed and divided into right and left halves. The left half of the brain was cut transversely into blocks approximately 0.5 cm thick and fixed by immersion in 4% buffered paraformaldehyde (PFA; ProSci Tech, Thuringowa, Qld Australia) for 3 days prior to embedding in paraffin. Subsequently, 10 μm coronal sections were obtained at the level of the subventricular zone (SVZ) and mounted on SuperFrost Plus slides (Thermoscientific, USA). Sections were coded and all analysis was carried out in the subcortical and periventricular white matter and SVZ at the level of the lateral ventricle (Sheep Ovis aries–Section 720) in 2 sections per animal and 3 fields of view per region on each section.

2.2.1. Single label immunohistochemistry

Sections were dewaxed and rehydrated in serial alcohols followed by a 15 min incubation in 0.1 M PBS containing 1% Triton-X 100. For laminin immmunohistochemistry, sections were rehydrated in 0.1 M PBS in 1% Triton-X 100 for 15 min. Antigen retrieval was carried out on sections incubated with Proteinase K (40 μg/mL) for 30 min at 37 °C and cooled for 20 min at room temperature. Sections were then rinsed in PBS, incubated in 0.3% H_2O_2 and 50% methanol for 15 min at room temperature to block endogenous peroxidase activity, incubated with 2% normal goat serum blocking buffer in 0.1 M PBS for 30 min at room temperature to block non-specific binding and then incubated overnight with anti-laminin rabbit polyclonal antibody (1:200; Novus Biologicas, USA). The sections were then washed, incubated with a secondary biotinylated goat anti-rabbit antibody (1:200; Vector Labs, USA) for 45 min followed by 45 min incubation with streptavidin horseradish