Chapter 16

Using Pregnant Sheep to Model Developmental Brain Damage

Lotte G. van den Heuij, Guido Wassink, Alistair J. Gunn, and Laura Bennet

Abstract

In order to develop more effective ways of identifying, managing, and treating preterm asphyxial brain injury, stable experimental models are essential. The present review describes the key experimental factors that determine the pattern and severity of brain injury in chronically instrumented fetal sheep, including the depth ("severity") and duration of asphyxia, and the maturity, and condition of the fetus. These models are valuable to dissect the pathogenesis of key clinical patterns of brain injury in a stable thermal and biochemical environment, and to test therapeutic interventions.

Key words Fetal sheep, Perinatal asphyxia, Hypoxic-ischemic encephalopathy, Hypotension, Cerebral blood flow

1 Introduction

Premature birth is one of the leading causes of morbidity and mortality. Around 6–13 % of all births are preterm [1]. Preterm survivors have a high risk of neurobehavioral disturbances and intellectual disabilities related to learning, cognition, visuospatial integration, attention deficit, and socialization [2–4]. Early imaging and postmortem data suggest that cerebral injury in preterm infants occurs in the immediate perinatal period in approximately two thirds of cases, while an appreciable number of cases occur before the onset of labor; in contrast, injury after the early neonatal period represents only approximately 10 % of cases [5, 6]. Consistent with this, acute EEG abnormalities are reported in the early perinatal period in the majority of infants and are highly predictive of long-term outcome [7]. The precise etiology of injury remains surprisingly unclear; however, it is highly likely to involve both hypoxia-ischemia and infection/inflammation [8].

David W. Walker (ed.), *Prenatal and Postnatal Determinants of Development*, Neuromethods, vol. 109,
DOI 10.1007/978-1-4939-3014-2_16,

Acute neonatal encephalopathy associated with asphyxia remains a significant cause of death and long-term disability at all ages [9]. Early onset neonatal encephalopathy is highly associated with evidence of asphyxia as shown by non-reassuring fetal heart rate tracings and severe metabolic acidosis on umbilical cord blood [10, 11], with acute brain lesions on magnetic resonance imaging, and ultimately with subsequent neurodevelopmental impairment [12]. In term infants, large clinical trials have established that mild brain cooling significantly reduces cerebral palsy and improves survival without disability at 18 months of age after acute hypoxic-ischemic encephalopathy [13]. However, therapeutic hypothermia is only partially protective, such that nearly half of affected babies still die or survive with disability despite treatment. Moreover, it is effective only within a relatively limited window of time, and current protocols may not be appropriate for preterm infants. There is a strong historical link between mild hypothermia and increased mortality in preterm newborns [14], and recently a phase 1 trial of cooling for preterm infants was stopped by the US Food and Drug Administration because of potential concerns about intracerebral hemorrhage. Thus, it is vital to find specific treatments for brain injury in the preterm brain.

Many drugs have been shown to be neuroprotective in neonatal rodent models; however, none have yet shown clinical improvement [15]. In order to translate promising treatments, it is essential to validate them in large animal translational models before considering clinical trials. Such models are also useful to help understand the many factors that affect outcome from an event, including the fetal sex, weight, and degree of hypotension.

1.1 What Initiates Neuronal Injury?

Before discussing the experimental approach, it is helpful to reflect on the triggers of asphyxial injury to the brain and other organs. Fundamentally, injury requires a period of insufficient oxygen and substrate delivery (glucose and other substances such as lactate) such that neurons (and glia) cannot maintain internal homeostasis and become depolarized [16]. At least broadly, the extent of injury is related to the duration of tissue depolarization [17]. Since organ perfusion is essential for maintaining oxygen and substrate delivery to tissues, not surprisingly, there is now considerable evidence to suggest that brain perfusion is the key factor that determines whether or not neural injury occurs after severe hypoxia/asphyxia [18–20]. Conversely, it is critical to appreciate that the fetus can fully adapt to mild to moderate reductions in oxygen tension without injury, from normal values of greater than 20 mmHg down to 10–12 mmHg [21]. Thus, in general, hypotension during a severe hypoxic challenge is a central requirement to reliably induce asphyxial neural injury.

Compression of the umbilical cord due to knots or entanglements of the cord or prolapse after rupture of the amniotic

membranes in labor are known potential causes of fetal asphyxia. Further, if amniotic fluid is reduced, e.g., in the growth retarded fetus, or lost after rupture of the amniotic membranes in an early stage of labor, or even worse, if the cord becomes trapped in the birth canal, physiologic uterine contractions can compress the umbilical cord between the fetus and uterine wall, giving rise to fetal hypoxemia, anaerobic glycolysis, and acidemia, with repeated or fixed deceleration of the fetal heart rate [22]. In view of these considerations, the present review focuses on induction of severe asphyxia using umbilical cord occlusion in preterm fetal sheep.

1.2 Choice of Experimental Species: Why Use the Sheep?

The species that is chosen for experimental studies must be appropriate to the scientific question. Rodent models are relatively cheap, and have an extensive repertoire of behavioral tests and molecular probes. However, such lissencephalic small animal species, with relatively small white matter tracts, are very unsatisfactory for studies of white matter injury and subsequent impaired cortical development. The absence of a gyral structure distorts the distribution of injury and makes it impossible to assess effects on cortical complexity. In contrast, the gyrencephalic structure of the fetal sheep brain, with large white matter volumes, is highly advantageous for studies of intracerebral white matter injury. No other experimental paradigms have so clearly mimicked the common pathological finding of diffuse white matter injury, with subcortical neural damage, as well as the model of asphyxia in the preterm fetal sheep [23, 24]. Equally important, the larger size of the fetal sheep enables intensive physiological, cerebrovascular and electrophysiological monitoring, which are not possible in newborn rodents. The relatively large size of the sheep fetus enables extensive implantation of instrumentation to allow continuous measurement of fetal heart rate, blood pressure (arterial and venous) [25], behavior (body movements) [26], blood flow to the brain and periphery and brain metabolism [27], intracerebral oxygenation as measured by near infrared spectroscopy [24], sympathetic activity, temperature [23, 28], and more. Catheters in the ewe and fetus also allow access for blood sampling, treatments and euthanasia. This comprehensive approach permits a significant physiological assessment of multiple organ systems in utero in a stable environment, without the confounding effects of anesthesia, ventilation, and changes in temperature.

2 Materials

2.1 Experimental Population and Procedures

Many potentially suitable breeds of sheep are available, although there is little information on whether this affects responses to asphyxia. For reference, our studies are currently conducted using pregnant Romney ewes, time-mated with Suffolk rams at

approximately 3–4 years of age. This breed has a typical gestational period of 145–147 days.

Sheep are highly precocial. Neural development of the fetal sheep at 0.8–0.85 of gestation is broadly similar to the full-term human infant [29, 30], while the 0.7 gestation fetus is broadly equivalent to the late preterm infant at 30–34 weeks, before the onset of cortical myelination, and at 0.6 gestation the sheep fetus is similar to the 26–28 week gestation human infant.

Experimentation should be begun 3–5 days after surgical instrumentation to allow fetal stress responses to resolve and for normal behavior to return. Ideally, all physiological data should be recorded continuously from 24 h or more before study and continued until the end of the study. Even if this is not possible, it is absolutely essential to display continuous ECG and blood pressure measurements during and immediately after umbilical cord occlusion to assess the onset and rate of fall of fetal blood pressure and to detect cardiac arrhythmias.

2.2 Experimental Procedures

2.2.1 Complete Occlusion of the Umbilical Cord

Complete compression of the umbilical cord is performed by inflating the occluder with a volume known to completely occlude the umbilical cord. This may be verified in pilot experiments with a Transonic flow probe placed around an umbilical vein [31]. Pragmatically, successful complete occlusion is can be routinely confirmed by rapid onset of fetal bradycardia and arterial hypertension, within the first minute. Blood composition measurements are taken at standard time points to assess fetal condition. Upon completion of the occlusion protocol the occluder is deflated. It is unknown whether rapid deflation of the occluder has any material effect on fetal recovery compared for example with controlled deflation over 10 s.

The total duration of occlusion required to induce hypotension and neural injury is a function of maturity, and may need to be fine-tuned in different settings or with different breeds of sheep. For reference, in our studies we found that the optimal durations of near-terminal single periods of complete occlusion were as follows: 30 min at 0.6 gestation [26, 31], 25 min at 0.7 gestation [27, 32, 33], and 15 min at 0.85 gestation [34–36].

2.2.2 Phases of Fetal Adaptation to Umbilical Cord Occlusion

As shown in Fig. 1, distinct phases of fetal adaptation may be clearly distinguished. There is an initial vagally mediated bradycardia, and an increase in mean arterial blood pressure, associated with intense peripheral vasoconstriction (the “compensation” phase). At this time carotid blood flow is maintained at around baseline values by vasoconstriction (Fig. 2), in contrast with an increase cerebral blood flow during moderate hypoxia [21]. During this phase EEG activity is profoundly suppressed [25, 31, 37], reducing metabolic demand. Microsphere studies in term fetal sheep have shown that although total brain flow does not change in this initial phase,

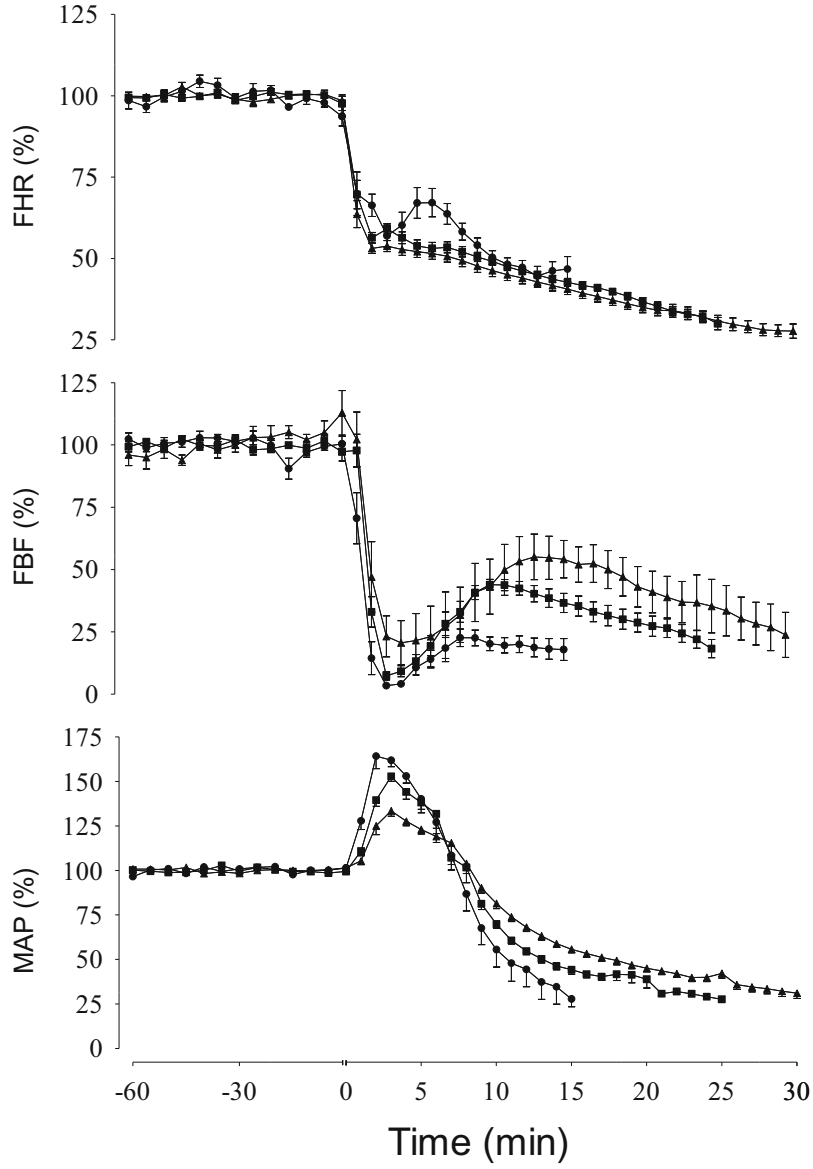

Fig. 1 Cardiovascular responses to prolonged umbilical cord occlusion in fetal sheep at 0.6, 0.7, or 0.85 gestation. Fetal heart rate (FHR, bpm, *top panel*), femoral blood flow (FBF, ml/min, *second panel*) and mean arterial pressure (MAP, mmHg, *bottom panel*) data represent 5 min averages before asphyxia, and 1 min averages during asphyxia and are expressed as percentages of baseline. The period of umbilical cord occlusion for each group starts at time zero; recovery data are not shown. Data are mean ± SE. Data modified from Wassink et al. [25]

fetal blood flow is redistributed within the brain, away from the cerebrum and choroid plexus, towards the brain stem [38].

As asphyxia continues these compensatory responses are attenuated (Figs. 1 and 2). There is a progressive fall in heart rate as

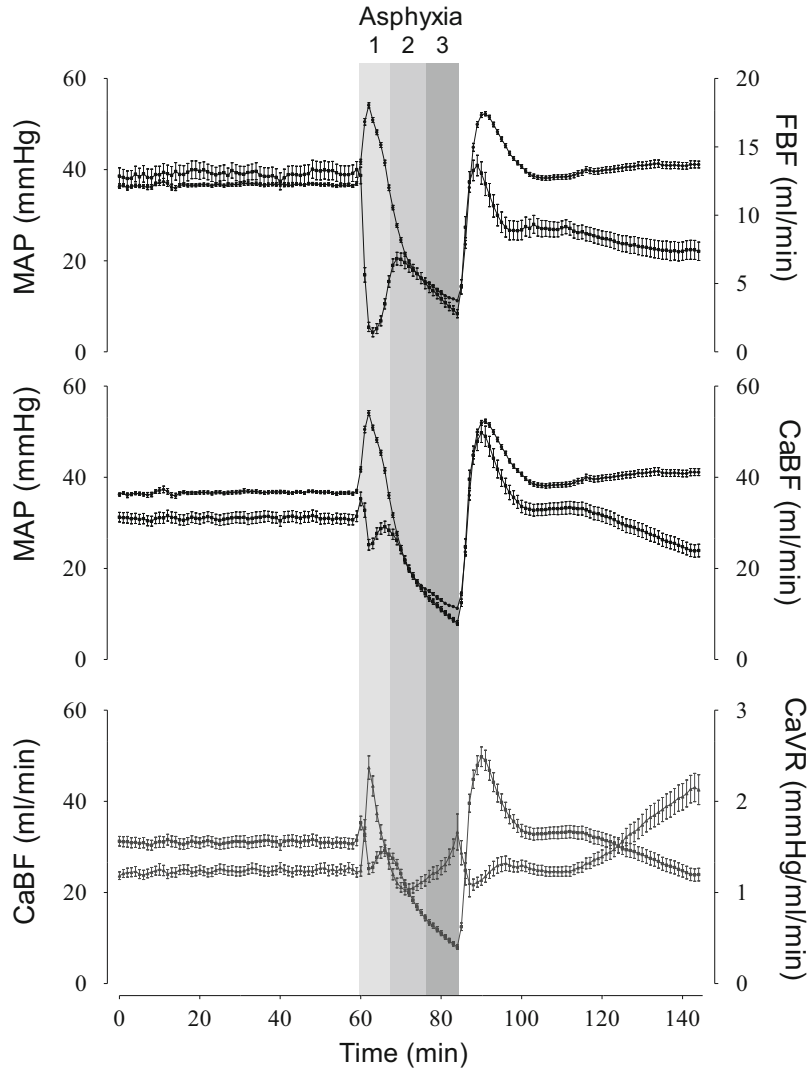

Fig. 2 Data before, during and after 15 min of complete umbilical cord occlusion in 0.7 gestation fetal sheep. 1 min averages ± SEM. The *top panel* shows time sequence changes in mean arterial blood pressure (MAP (*filled circle*, *left axis*)) and femoral blood flow (FBF (*filled square*, *right axis*)), the *middle panel* shows MAP (*filled circle*, *left axis*) and carotid blood flow (CaBF (*filled square*, *right axis*)) and the *bottom panel* shows carotid blood flow (*filled square*, *left axis*) and carotid vascular resistance (CaVR (*filled triangle*, *right axis*)). The asphyxial period is broken into three phases: compensation (1), the start of decompensation (2), with pressure passive blood flow, and the final stages of decompensation (3), where the fall in blood pressure is slowed by increased central and peripheral vascular resistance, which in turn leads to further compromised brain and peripheral organ perfusion

cardiac function becomes impaired secondary to hypoxia, acidosis, depletion of myocardial glycogen, and cardiomyocyte injury (Fig. 1) [39]. Peripheral vasoconstriction, which had functioned to maintain blood pressure in the face of the profound

bradycardia, is lost with partial or near complete vasodilatation occurring depending on the vascular bed in question (Figs. 1 and 2) [25, 40]. Recent telemetry studies of renal sympathetic nerve activity show that failure to maintain vascular resistance is at least in part related to a failure to maintain neural control of peripheral vascular tone [41].

Once MAP falls below baseline in a so-called "decompensation" phase [22, 25, 40], cerebral perfusion falls in parallel, consistent with the known relatively narrow low range of autoregulation of cerebral blood flow in the fetus (Fig. 2) [42]. This loss of autoregulation has been proposed to be a key factor mediating injury, particularly in preterm infants [40]. However, experimental data show that loss of peripheral vascular resistance is not complete. As Fig. 2 shows, in preterm fetal sheep, peripheral vasoconstriction begins to be attenuated around 4–5 min of asphyxia, in association with a progressive fall in arterial blood pressure. Cerebral perfusion falls below baseline values from around 7–8 min, and there is partial restoration of peripheral perfusion that is greatest at 12 min (Fig. 2). However, the precipitous fall in blood pressure is attenuated through by stabilisation of the cerebral and peripheral vascular resistance at baseline values from around 12 min. The effect of this shown by slowing of the fall in blood pressure after about 15 min, such that perfusion no longer mirrors the fall in pressure.

The observation that the preterm fetus is able to partially maintain vascular resistance throughout prolonged complete umbilical cord occlusion [25], argues that total loss of autoregulator control is not required for injury. Further research is required to define the role of autoregulation in the processes mediating injury. The redistribution of blood flow to the brainstem is likely progressively lost, although this has not been extensively quantified for the term or preterm fetus. Given that the preterm fetus can survive asphyxia for longer and is thus exposed to a greater period of hypoperfusion, this likely underpins injury to brainstem regions [26, 43].

2.2.3 Prolonged Partial Umbilical Cord Occlusion

Prolonged periods of partial asphyxia in utero can be produced by partial compression of the umbilical cord. Partial cord compression for 90 min induced severe asphyxial insults in near-term fetal sheep [44], similar in nature to those following uterine hypoperfusion [45] except for some relatively minor haemodynamic differences, followed by delayed development of seizures. Although this approach might seem to be less controllable than occlusion of the uterine blood supply, in practice it is easy to vary the degree of occlusion, and umbilical venous flow can be measured directly with an ultrasonic flow probe. Both methods produce similar levels of asphyxia and evidence of encephalopathy with typically relatively variable neural injury [44, 46].

3 Technical Notes

Umbilical cord occlusion is a very simple experimental technique. The most common experimental problem is failure to recovery after release of occlusion. In term fetuses, moderate insults, such as 10 min of complete umbilical cord occlusion in the near-term fetal sheep, are associated with little risk of mortality in healthy fetuses, who then go on to develop selective neuronal loss [37, 47]. In contrast, more prolonged, complete occlusion is intrinsically associated with the development of severe hypotension [25, 35, 36, 48].

Successful recovery from complete occlusion is confirmed by a rapid, overshoot increase of FHR and MAP. If bradycardia persists for more than 30 s or blood pressure does not increase to over 50 % of baseline in the first 60 s after release of occlusion then a dose of epinephrine (0.1 ml/kg estimated weight, 1:10,000 epinephrine) should be given to the fetus via the brachial vein by slow i.v. push. Even before this stage, fetal cardiovascular status can sometimes be stimulated using a non-pharmaceutical method of making the ewe move abruptly, by nudging or generally touching her flank or back for example. This can have the effect of promoting a changing fetal position and rapidly improving fetal perfusion. More often than not, this facilitates resuscitation such that epinephrine may not be required.

Fetal death may be associated either with asystole or with more rapid onset of hypotension as previously described [26, 49]. Fortunately, because hypotension develops progressively, it is possible to identify warning signs such as more rapid fall in blood pressure, or marked blood pressure or heart rate instability before it becomes terminal by continuously monitoring fetal arterial blood pressure. If the goal of the study is to test post-insult treatment it may be reasonable to stop occlusion a little earlier (1–2 min) in such cases to avoid cardiac death, provided that the experimenters use consistent criteria and robustly randomize all fetuses between groups before occlusion.

Examples of such criteria are the level to which blood pressure falls beyond which the fetus is seldom able to reestablish perfusion. In the preterm, 0.7 gestation fetus, for example, our experience suggests it is appropriate to stop occlusion when mean arterial blood pressure falls from baseline values of 35–40 mmHg down to a cut-off level of 8 mmHg or below. Equally, occlusion should stop if there is fetal asystole or arrhythmia. Even before this point, we observed that fetuses that reached mean arterial blood pressure below 15 mmHg by 15 min of occlusion reached the terminal cut-off blood pressure before 25 min, thus giving the experimenter early warning of impending problems [49].

4 Determinants of Outcome

4.1 Sensitisation and Tolerance to Injury

It is widely speculated that adverse conditions such preexisting hypoxia and infection may impair the ability of the fetus to mount an effective defense response to hypoxia and render neurons and glia more sensitive to hypoxia [50]. The most common metabolic disturbance to the fetus is intrauterine growth retardation (IUGR) associated with placental dysfunction. Indeed, mild maternal undernutrition that does not alter fetal growth may still affect development of the fetal hypothalamic–pituitary–adrenal function, with reduced pituitary and adrenal responsiveness to moderate hypoxia [51]. Clinically, overt IUGR is usually associated with a greater risk of brain injury, albeit the risks have fallen over time [10]. Consistent with this observation, chronically hypoxic fetuses from multiple pregnancies developed much more severe, progressive metabolic acidosis than previously normoxic fetuses during brief (1 min) umbilical cord occlusions repeated every 5 min (pH 7.07 ± 0.14 vs. 7.34 ± 0.07) and hypotension (a nadir of 24 ± 2 mmHg vs. 45.5 ± 3 mmHg after 4 h of repeated occlusion) [52]. Fetuses with preexisting hypoxia were smaller on average, and had lower blood glucose values and higher $PaCO_2$ values. Similarly, in normally grown fetuses, 5 days of induced chronic hypoxemia was associated with increased striatal damage after acute exposure to repeated umbilical cord occlusion for 5 min every 30 min for a total of four occlusions [53]. Together, these data support the clinical concept that fetuses with chronic placental insufficiency are vulnerable even to relatively infrequent periods of brief asphyxia in early labor. However, under some circumstances at least, this appears not to be the case. In spontaneously hypoxic fetuses, we have observed, for example, that the initial chemoreflex response to asphyxia induced by umbilical cord occlusion is enhanced [48].

Exposure to the endotoxin lipopolysaccharide (LPS) can cause neural injury [54–56]. We have recently shown, rather intriguingly, that exposure to asphyxia after 4 days of chronic LPS exposure actually reduced white matter injury in the preterm fetal sheep [56]. However, timing between insults is important. Studies in newborn rats have shown a single injection of LPS (which does not normally cause injury) given within 6 h or more than 72 h before an hypoxic-ischemic insult (carotid artery ligation plus hypoxia) increases neural injury [57, 58]. In contrast, injection of LPS at intermediate times such as 24 h before hypoxia-ischemia reduced subsequent neural injury, i.e., preconditioned the brain [58]. The critical effect of timing suggests that LPS exposure protection requires transcriptional changes. For example, a recent study suggests that the transcription factor nuclear factor E2-related factor 2, and the transcription cofactor, peroxisome proliferator-activated

receptor-γ coactivator 1α play a key role in mediating LPS induced neuroprotection in the neonatal rat [59].

4.2 Brain Maturity

Maturation of the brain and body have dramatic and underappreciated effects on cardiac and neural sensitivity to asphyxia [25, 26, 60]. Boyle's experiments with Hooke and others in the seventeenth century, elegantly demonstrated that younger animals have much greater tolerance to hypoxia [61]. Extensive work by perinatal scientists such as Dawes, Mott, and Heather in the 1950s and 1960s demonstrated that anaerobic capacity during fetal life underpinned greater fetal tolerance to hypoxia. We and others have shown that the younger the fetus, the greater the tolerance to hypoxia [62]. For example, the premature sheep fetus at 90 days gestation (term is 147 days), prior to the onset of cortical myelination, can tolerate extended periods of up to 20 min of umbilical cord occlusion without neuronal loss [26, 31, 60]. As Fig. 1 demonstrates, the initial adaptation to severe hypoxia, is consistent at all ages, but the critical difference is that the preterm fetus can keep its heart going for longer and thus, the younger the fetus, the longer the survival time.

This is consistent with the observation by Shelley that cardiac glycogen peaks during preterm-equivalent life in a wide variety of mammalian species [63]. In the fetal sheep for example, at 0.6 gestation the majority of fetuses survived up to 30 min of complete umbilical cord occlusion and did not require resuscitation with epinephrine and did not develop cardiac injury at 3 days post-insult [31]. In contrast, near-term fetuses can tolerate 10–12 min, typically without requiring resuscitation [64]. A near-terminal insult is around 15–18 min, with most fetuses requiring resuscitation, and showing subsequent severe cardiac dysfunction [25, 35, 39, 65, 66].

Critically, as a consequence of this extended survival during severe asphyxia, the premature fetus is exposed to extremely prolonged and profound hypotension and hypoperfusion. At 0.6 gestation, for example, no injury occurs after 20 min of complete umbilical cord occlusion even though hypotension is already present (Fig. 1) [26, 60], but severe subcortical injury occurs if the occlusion is continued for 30 min [26]. Speculatively, there may be failure of redistribution of blood flow within the fetal brain during the phase of severe hypotension, which places previously protected areas of the brain such as the brainstem at risk of injury [67].

4.2.1 The Effect of Fetal sex

Numerous studies have confirmed that there is an increased risk of perinatal mortality and morbidity in boys compared to girls at all stages of gestation [68]. The mechanisms mediating the influence of gender on perinatal death and disability are poorly understood and likely to be multifactorial. There is increasing evidence in the developing brain that estradiol may play a neuroprotective role [69]. There is evidence of sex-related differences in the pathways

leading to apoptosis [70], and in cell sensitivity to excitotoxins [70, 71]. Recent data in human infants shows sex-related differences in the CSF levels of IL-8 and antioxidants after asphyxia, with higher levels in newborn females that may contribute to the greater vulnerability to brain injury in males than females [72].

There are also data that male fetuses may be less able to adapt to hypoxic stress. Males fetuses have higher rates of abnormal fetal heart rate recordings, metabolic acidosis, and need for operative intervention or resuscitation in labor [73–77]. Male fetuses are on average bigger, grow faster, and have a higher metabolic rate than females [78, 79], suggesting that when oxygen is limited they might deplete available resources more rapidly. Further, there is evidence that males have relatively delayed maturation of some aspects of autonomic nervous system function, such as for example, adrenal medullary and lung beta-receptor maturation in fetal rabbits [80]. Clinically, after exposure to asphyxia at birth preterm boys are reported to have lower plasma catecholamine levels than girls [81]. Moreover, data from pregnancies complicated by placental insufficiency before 34 weeks, showed that cardiac tropin levels are significantly greater in male than female fetuses [82].

The sex of the fetus per se did not significantly alter the cardiovascular responses of healthy singleton preterm (0.7 gestation) fetal sheep to an acute, profound asphyxial insult [49]. Neither the average responses, nor the incidence or timing of failure to complete the full period of umbilical cord occlusion were significantly different between the sexes. However, overall, significantly more male fetuses developed profound hypotension (<8 mmHg) before the end of the occlusion period. Further, blood pressure at 15 min correlated with fetal weight for male fetuses, but not female fetuses suggesting a role for metabolic reserve in facilitating this capacity [49]. These data further support the idea that metabolic substrate availability has a greater impact on male fetuses.

The causes of failure to complete the full target duration of occlusions differed markedly between male and female sheep fetuses. These differences were associated in turn with changes that suggest altered chemoreflex and cardiac responses between the genders. The short-occlusion-males demonstrated slower and reduced initial peripheral vasoconstriction compared with the full-occlusion fetuses. This was followed by earlier and significantly greater hypotension, associated with greater falls in heart rate and carotid and femoral blood flow. In contrast, short-occlusion-females showed a markedly more rapid onset of initial vasoconstriction of the femoral bed, and subsequent falls in blood pressure and heart rate that were intermediate between the full-occlusion fetuses and short-occlusion-males. It is improbable that these differences relate to placental function, since fetal body weight (measured 3 days after occlusion) and pH, blood gas, glucose and lactate values before occlusion were not different between the groups.

5 Final Conclusions

The experimental models outlined in this chapter will continue to be developed and refined, aiming to mimic human pathophysiology as closely as possible at the whole body level, while allowing the factors contributing to the wide variability in outcomes observed after perinatal asphyxia to be dissected. Both systemic and cerebral factors are important in real life, and include respectively the determinants of cardiovascular decompensation and factors that modulate the intrinsic vulnerability of the brain, such as environmental temperature, metabolic status and expression of neurotrophic factors. In turn, these factors are highly likely to affect responses to treatment. As the critical events which precipitate significant perinatal hypoxic-ischaemic encephalopathy are better understood, our ability to identify and intervene in clinical asphyxia will also improve.

Acknowledgments

The authors' work reported in this review has been supported by the Health Research Council of New Zealand, Lottery Health Board of New Zealand, the Auckland Medical Research Foundation, the National Institutes of Health, and the March of Dimes Birth Defects Trust.

References

1. Committee on Understanding Premature Birth and Assuring Healthy Outcomes. Preterm birth: causes, consequences, and prevention. In: Behrman RE, Butler AS (eds). Washington DC: Institute of Medicine of the National Academies, 2007. http://books.nap.edu/openbook.php?record_id=11622&page=1. Accessed on 1 March 2013
2. Mullen KM, Vohr BR, Katz KH et al (2011) Preterm birth results in alterations in neural connectivity at age 16 years. Neuroimage 54:2563–2570
3. Marlow N, Hennessy EM, Bracewell MA et al (2007) Motor and executive function at 6 years of age after extremely preterm birth. Pediatrics 120:793–804
4. Rogers CE, Anderson PJ, Thompson DK et al (2012) Regional cerebral development at term relates to school-age social-emotional development in very preterm children. J Am Acad Child Adolesc Psychiatry 51:181–191
5. de Vries LS, Eken P, Groenendaal F et al (1998) Antenatal onset of haemorrhagic and/or ischaemic lesions in preterm infants: prevalence and associated obstetric variables. Arch Dis Child Fetal Neonatal Ed 78:F51–F56
6. Bell JE, Becher JC, Wyatt B et al (2005) Brain damage and axonal injury in a Scottish cohort of neonatal deaths. Brain 128:1070–1081
7. Kubota T, Okumura A, Hayakawa F et al (2002) Combination of neonatal electroencephalography and ultrasonography: sensitive means of early diagnosis of periventricular leukomalacia. Brain Dev 24:698–702
8. Perlman JM (1998) White matter injury in the preterm infant: an important determination of abnormal neurodevelopment outcome. Early Hum Dev 53:99–120
9. Gunn AJ, Gunn TR (1997) Changes in risk factors for hypoxic-ischaemic seizures in term infants. Aust N Z J Obstet Gynaecol 37:36–39
10. Westgate JA, Gunn AJ, Gunn TR (1999) Antecedents of neonatal encephalopathy with fetal acidaemia at term. Br J Obstet Gynaecol 106:774–782

11. Wyatt JS, Gluckman PD, Liu PY et al (2007) Determinants of outcomes after head cooling for neonatal encephalopathy. Pediatrics 119: 912–921
12. MacLennan A, The International Cerebral Palsy Task Force, Gunn AJ et al (1999) A template for defining a causal relation between acute intrapartum events and cerebral palsy: international consensus statement. BMJ 319:1054–1059
13. Edwards AD, Brocklehurst P, Gunn AJ et al (2010) Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. BMJ 340: c363
14. Gunn AJ, Bennet L (2008) Brain cooling for preterm infants. Clin Perinatol 35:735–748
15. Robertson NJ, Tan S, Groenendaal F et al (2012) Which neuroprotective agents are ready for bench to bedside translation in the newborn infant? J Pediatr 160:544–552.e544
16. Wassink G, Gunn ER, Drury PP et al (2014) The mechanisms and treatment of asphyxial encephalopathy. Front Neurosci 8:40
17. Dijkhuizen RM, Beekwilder JP, van der Worp HB et al (1999) Correlation between tissue depolarizations and damage in focal ischemic rat brain. Brain Res 840:194–205
18. Gunn AJ, Parer JT, Mallard EC et al (1992) Cerebral histologic and electrocorticographic changes after asphyxia in fetal sheep. Pediatr Res 31:486–491
19. Mallard EC, Williams CE, Johnston BM et al (1994) Increased vulnerability to neuronal damage after umbilical cord occlusion in fetal sheep with advancing gestation. Am J Obstet Gynecol 170:206–214
20. Fujii EY, Takahashi N, Kodama Y et al (2003) Hemodynamic changes during complete umbilical cord occlusion in fetal sheep related to hippocampal neuronal damage. Am J Obstet Gynecol 188:413–418
21. Giussani DA, Spencer JAD, Hanson MA (1994) Fetal and cardiovascular reflex responses to hypoxaemia. Fetal Matern Med Rev 6:17–37
22. Westgate JA, Wibbens B, Bennet L et al (2007) The intrapartum deceleration in center stage: a physiological approach to interpretation of fetal heart rate changes in labor. Am J Obstet Gynecol 197:e1–e11.236
23. Bennet L, Roelfsema V, George S et al (2007) The effect of cerebral hypothermia on white and grey matter injury induced by severe hypoxia in preterm fetal sheep. J Physiol 578:491–506
24. Bennet L, Roelfsema V, Pathipati P et al (2006) Relationship between evolving epileptiform activity and delayed loss of mitochondrial activity after asphyxia measured by near-infrared spectroscopy in preterm fetal sheep. J Physiol 572:141–154
25. Wassink G, Bennet L, Booth LC et al (2007) The ontogeny of hemodynamic responses to prolonged umbilical cord occlusion in fetal sheep. J Appl Physiol 103:1311–1317
26. George S, Gunn AJ, Westgate JA et al (2004) Fetal heart rate variability and brainstem injury after asphyxia in preterm fetal sheep. Am J Physiol Regul Integr Comp Physiol 287: R925–R933
27. Bennet L, Roelfsema V, Dean J et al (2007) Regulation of cytochrome oxidase redox state during umbilical cord occlusion in preterm fetal sheep. Am J Physiol Regul Integr Comp Physiol 292:R1569–R1576
28. Gunn AJ, Gunn TR, de Haan HH et al (1997) Dramatic neuronal rescue with prolonged selective head cooling after ischemia in fetal lambs. J Clin Invest 99:248–256
29. Barlow RM (1969) The foetal sheep: morphogenesis of the nervous system and histochemical aspects of myelination. J Comp Neurol 135:249–262
30. McIntosh GH, Baghurst KI, Potter BJ et al (1979) Foetal brain development in the sheep. Neuropathol Appl Neurobiol 5: 103–114
31. Bennet L, Rossenrode S, Gunning MI et al (1999) The cardiovascular and cerebrovascular responses of the immature fetal sheep to acute umbilical cord occlusion. J Physiol 517: 247–257
32. Quaedackers JS, Roelfsema V, Heineman E et al (2004) The role of the sympathetic nervous system in post-asphyxial intestinal hypoperfusion in the preterm sheep fetus. J Physiol 557:1033–1044
33. Quaedackers JS, Roelfsema V, Hunter CJ et al (2004) Polyuria and impaired renal blood flow after asphyxia in preterm fetal sheep. Am J Physiol Regul Integr Comp Physiol 286: R576–R583
34. Wibbens B, Westgate J, Bennet L, et al. The relationship between changes in the ST complex and the development of hypotension during prolonged asphyxia in the near term fetal sheep. Paper presented at Proceedings, IUPS Fetal Physiology Satellite Meeting, 2001, Auckland, New Zealand
35. Wibbens B, Westgate JA, Bennet L et al (2005) Profound hypotension and associated ECG changes during prolonged cord occlusion in

the near term fetal sheep. Am J Obstet Gynecol 193:803–810

36. Drury PP, Davidson JO, van den Heuij LG et al (2014) Status epilepticus after prolonged umbilical cord occlusion is associated with greater neural injury fetal sheep at term-equivalent. PLoS One 9:e96530
37. Hunter CJ, Bennet L, Power GG et al (2003) Key neuroprotective role for endogenous adenosine A1 receptor activation during asphyxia in the fetal sheep. Stroke 34:2240–2245
38. Jensen A, Hohmann M, Kunzel W (1987) Dynamic changes in organ blood flow and oxygen consumption during acute asphyxia in fetal sheep. J Dev Physiol 9:543–559
39. Gunn AJ, Maxwell L, de Haan HH et al (2000) Delayed hypotension and subendocardial injury after repeated umbilical cord occlusion in near-term fetal lambs. Am J Obstet Gynecol 183:1564–1572
40. Bennet L, Booth LC, Drury PP et al (2012) Preterm neonatal cardiovascular instability: does understanding the fetus help evaluate the newborn? Clin Exp Pharmacol Physiol 39:965–972
41. Booth LC, Malpas SC, Barrett CJ et al (2012) Renal sympathetic nerve activity during asphyxia in fetal sheep. Am J Physiol Regul Integr Comp Physiol 303:R30–R38
42. Parer JT (1998) Effects of fetal asphyxia on brain cell structure and function: limits of tolerance. Comp Biochem Physiol A Mol Integr Physiol 119:711–716
43. Logitharajah P, Rutherford MA, Cowan FM (2009) Hypoxic-ischemic encephalopathy in preterm infants: antecedent factors, brain imaging, and outcome. Pediatr Res 66:222–229
44. Ball RH, Espinoza MI, Parer JT et al (1994) Regional blood flow in asphyxiated fetuses with seizures. Am J Obstet Gynecol 170:156–161
45. Ikeda T, Murata Y, Quilligan EJ et al (1998) Fetal heart rate patterns in postasphyxiated fetal lambs with brain damage. Am J Obstet Gynecol 179:1329–1337
46. Ikeda T, Murata Y, Quilligan EJ et al (1998) Physiologic and histologic changes in near-term fetal lambs exposed to asphyxia by partial umbilical cord occlusion. Am J Obstet Gynecol 178:24–32
47. Mallard EC, Williams CE, Johnston BM et al (1995) Neuronal damage in the developing brain following intrauterine asphyxia. Reprod Fertil Dev 7:647–653
48. Wibbens B, Bennet L, Westgate JA et al (2007) Pre-existing hypoxia is associated with a delayed but more sustained rise in T/QRS ratio during prolonged umbilical cord occlusion in near-term fetal sheep. Am J Physiol Regul Integr Comp Physiol 293: R1287–R1293
49. Bennet L, Booth LC, Ahmed-Nasef N et al (2007) Male disadvantage? Fetal sex and cardiovascular responses to asphyxia in preterm fetal sheep. Am J Physiol Regul Integr Comp Physiol 293:R1280–R1286
50. Gunn AJ, Bennet L (2009) Fetal hypoxia insults and patterns of brain injury: insights from animal models. Clin Perinatol 36:579–593
51. Hawkins P, Steyn C, McGarrigle HH et al (2000) Effect of maternal nutrient restriction in early gestation on responses of the hypothalamic-pituitary-adrenal axis to acute isocapnic hypoxaemia in late gestation fetal sheep. Exp Physiol 85:85–96
52. Westgate J, Wassink G, Bennet L et al (2005) Spontaneous hypoxia in multiple pregnancy is associated with early fetal decompensation and greater T wave elevation during brief repeated cord occlusion in near-term fetal sheep. Am J Obstet Gynecol 193:1526–1533
53. Pulgar VM, Zhang J, Massmann GA et al (2007) Mild chronic hypoxia modifies the fetal sheep neural and cardiovascular responses to repeated umbilical cord occlusion. Brain Res 1176:18–26
54. Dean JM, van de Looij Y, Sizonenko SV et al (2011) Delayed cortical impairment following lipopolysaccharide exposure in preterm fetal sheep. Ann Neurol 70:846–856
55. Mathai S, Booth LC, Davidson JO et al (2013) Acute on chronic exposure to endotoxin in preterm fetal sheep. Am J Physiol Regul Integr Comp Physiol 304:R189–R197
56. van den Heuij LG, Mathai S, Davidson JO et al (2014) Synergistic white matter protection with acute-on-chronic endotoxin and subsequent asphyxia in preterm fetal sheep. J Neuroinflammation 11:89
57. Yang L, Sameshima H, Ikeda T et al (2004) Lipopolysaccharide administration enhances hypoxic-ischemic brain damage in newborn rats. J Obstet Gynaecol Res 30:142–147
58. Eklind S, Mallard C, Arvidsson P et al (2005) Lipopolysaccharide induces both a primary and a secondary phase of sensitization in the developing rat brain. Pediatr Res 58:112–116
59. Correa F, Ljunggren E, Patil J et al (2013) Time-dependent effects of systemic lipopolysaccharide injection on regulators of antioxidant defence Nrf2 and PGC-1alpha in the neonatal rat brain. Neuroimmunomodulation 20:185–193

60. Keunen H, Blanco CE, van Reempts JL et al (1997) Absence of neuronal damage after umbilical cord occlusion of 10, 15, and 20 minutes in midgestation fetal sheep. Am J Obstet Gynecol 176:515–520
61. Boyle R (1670) New pneumatical experiments about respiration. Phil Trans Roy Soc Lond 62:2011–2031
62. Gunn AJ, Quaedackers JS, Guan J et al (2001) The premature fetus: not as defenseless as we thought, but still paradoxically vulnerable? Dev Neurosci 23:175–179
63. Shelley HJ (1961) Glycogen reserves and their changes at birth and in anoxia. Br Med Bull 17:137–143
64. Mallard EC, Gunn AJ, Williams CE et al (1992) Transient umbilical cord occlusion causes hippocampal damage in the fetal sheep. Am J Obstet Gynecol 167:1423–1430
65. Ley D, Oskarsson G, Bellander M et al (2004) Different responses of myocardial and cerebral blood flow to cord occlusion in exteriorized fetal sheep. Pediatr Res 55:568–575
66. Drury PP, Booth LC, Bennet L et al (2013) Dopamine infusion for post-resuscitation blood pressure support after profound asphyxia in near-term fetal sheep. Exp Physiol 98:699–709
67. Barkovich AJ, Sargent SK (1995) Profound asphyxia in the premature infant: imaging findings. AJNR Am J Neuroradiol 16:1837–1846
68. Joseph KS, Wilkins R, Dodds L et al (2005) Customized birth weight for gestational age standards: perinatal mortality patterns are consistent with separate standards for males and females but not for blacks and whites. BMC Pregnancy Childbirth 5:3
69. McCarthy MM (2008) Estradiol and the developing brain. Physiol Rev 88:91–134
70. Renolleau S, Fau S, Charriaut-Marlangue C (2008) Gender-related differences in apoptotic pathways after neonatal cerebral ischemia. Neuroscientist 14:46–52
71. Johnston MV, Hagberg H (2007) Sex and the pathogenesis of cerebral palsy. Dev Med Child Neurol 49:74–78
72. Hussein MH, Daoud GA, Kakita H et al (2007) The sex differences of cerebrospinal fluid levels of interleukin 8 and antioxidants in asphyxiated newborns. Shock 28:154–159
73. Dawes NW, Dawes GS, Moulden M et al (1999) Fetal heart rate patterns in term labor vary with sex, gestational age, epidural analgesia, and fetal weight. Am J Obstet Gynecol 180:181–187
74. Ingemarsson I, Herbst A, Thorngren-Jerneck K (1997) Long term outcome after umbilical artery acidaemia at term birth: influence of gender and duration of fetal heart rate abnormalities. Br J Obstet Gynaecol 104: 1123–1127
75. Bekedam DJ, Engelsbel S, Mol BW et al (2002) Male predominance in fetal distress during labor. Am J Obstet Gynecol 187: 1605–1607
76. Sheiner E, Levy A, Katz M et al (2004) Gender does matter in perinatal medicine. Fetal Diagn Ther 19:366–369
77. Thorngren-Jerneck K, Herbst A (2001) Low 5-minute Apgar score: a population-based register study of 1 million term births. Obstet Gynecol 98:65–70
78. Clarke CA, Mittwoch U (1995) Changes in the male to female ratio at different stages of life. Br J Obstet Gynaecol 102:677–679
79. Mittwoch U (2004) The elusive action of sex-determining genes: mitochondria to the rescue? J Theor Biol 228:359–365
80. Padbury JF, Hobel CJ, Lam RW et al (1981) Sex differences in lung and adrenal neurosympathetic development in rabbits. Am J Obstet Gynecol 141:199–204
81. Greenough A, Lagercrantz H, Pool J et al (1987) Plasma catecholamine levels in preterm infants. Effect of birth asphyxia and Apgar score. Acta Paediatr Scand 76:54–59
82. Nomura RM, Ortigosa C, Fiorelli LR et al (2011) Gender-specific differences in fetal cardiac troponin T in pregnancies complicated by placental insufficiency. Gend Med 8:202–208