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Animal models of hypoxic-ischemic encephalopathy: optimal choices for the best outcomes

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Abstract: Hypoxic-ischemic encephalopathy (HIE), a serious disease leading to neonatal death, is becoming a key area of pediatric neurological research. Despite remarkable advances in the understanding of HIE, the explicit pathogenesis of HIE is unclear, and well-established treatments are absent. Animal models are usually considered as the first step in the exploration of the underlying disease and in evaluating promising therapeutic interventions. Various animal models of HIE have been developed with distinct characteristics, and it is important to choose an appropriate animal model according to the experimental objectives. Generally, small animal models may be more suitable for exploring the mechanisms of HIE, whereas large animal models are better for translational studies. This review focuses on the features of commonly used HIE animal models with respect to their modeling strategies, merits, and shortcomings, and associated neuropathological changes, providing a comprehensive reference for improving existing animal models and developing new animal models.

Keywords: animal models; histological changes; hypoxic-ischemic encephalopathy; neonatal.

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Introduction

Approximately 45% of all annual deaths among children younger than 5 years of age are in newborns. The primary causes of neonatal death include prematurity, low birth weight, infections, asphyxia, and birth trauma, which account for nearly 80% of deaths in this age group (WHO, 2016). Neonatal encephalopathy follows birth trauma and asphyxia as the third most common cause of death among children younger than 5 years of age, accounting for 643 765 deaths globally in 2013 (Kyu et al., 2016). Perinatal hypoxic-ischemic encephalopathy (HIE), the dominant type of neonatal encephalopathy, remains an important clinical challenge for doctors. For every 1000 live births, the rate of morbidity from HIE ranges from 1 to 8 in developed countries and up to 26 in developing countries (Kurinczuk et al., 2010). Globally, 10–60% of infants with HIE will die and at least 25% of the surviving children will present with persistent neurological defects, including epilepsy, mental retardation, cerebral palsy, visual and hearing problems, cognitive disorders, and other neurophysiologic handicaps, which will impose a heavy burden on society and the families of the patients (Selway, 2010). Currently, moderate hypothermia (33–34°C) is used to treat moderate to severe HIE in many neonatal care centers. This treatment has been shown to be effective in newborns, reducing the risk of death or disability at 18–24 months of age and improving neurocognitive outcomes in middle childhood (Shankaran et al., 2005; Guillet et al., 2012; Jacobs et al., 2013). In spite of the benefits of cooling treatment, approximately 25% of infants die, and 20% of the survivors live with sensorimotor or cognitive impairments (Azzopardi, 2014). Moreover, there are still several unanswered questions regarding therapeutic hypothermia, such as the optimal timing for the initiation of hypothermia, the depth and duration of the hypothermia, and the speed of rewarming. Additional effective treatments to augment the protective effects of hypothermia also need to be developed (Gonzales-Portillo et al., 2014; Dixon et al., 2015).

Prenatal severe hypoxia-ischemia usually induces cortical and white matter damage in the brain. White matter damage, such as periventricular leukomalacia and periventricular white matter injury, is most frequent in preterm babies, especially in those of extremely low birth weights. Ninety percent of the babies with extremely low birth weights display cerebral palsy symptoms in childhood. In contrast, cortical injury is more common in full-term infants (Back, 2006). The pathogenesis of white matter injury, which is the dominant brain injury in preterm infants, is distinct from the pathogenesis observed in full-term infants. In addition to hypoxia-ischemia, systemic infection, and inflammation, the intrinsic vulnerability of developing oligodendrocytes also contributes to cerebral white matter injury in preterm infants (Volpe et al., 2011).

Animal models are considered as the first step in exploring mechanisms underlying primary disease and in assessing the safety and efficacy of treatments. Ideal animal models need to mimic the human HIE pathophysiological condition as much as possible, which is crucial for understanding the pathogenesis of HIE and for the proper assessment of promising pre-clinical treatments. Currently, a number of species are used as models to recapitulate different aspects of human HIE, including rodents (Craig, et al., 2003), piglets (Larson et al., 2013), rabbits (Derrick, 2004), sheep (Nitsos et al., 2014), and non-human primates (Juul et al., 2007). However, multiple organs are injured in the process of hypoxia-ischemia, which makes it difficult to produce ideal animal models that completely mimic the pathophysiological changes observed in human HIE. Moreover, promising therapeutic interventions in animal models may be ineffective or even harmful in humans. This review highlights the modeling methods used in the most common models of HIE and describes the characteristics of each model in order to provide a perspective for further research and the development of new models.

Animal models of HIE

Although the general development of the nervous system is similar among different species, differences in the time scales for the emergence of critical events also exist. Given that the sensitivity of the developing brain to environmental insults is time and region dependent, it is necessary to delineate the human equivalent of the time scale for brain development accurately in animal models (Table 1).

Table 1: Time scale of brain development in animals equivalent to human.

Species		Age of animal (days)	Human equivalent (gestational weeks)
Rat		P2–P5	24–32
		P7	32–34
		P10	Term newborn
Piglet		At birth	36–38
Rabbit		G22	22–27
Sheep		G95	24–28
		G135	Term newborn
Non-human primate	Rhesus monkey	G124	39
	Baboon	G125	26–28
		G140	30–32
		G160	Term newborn

P, Postnatal; G, gestational.

Rodents

Rat

The rat, the most commonly used animal model for HIE, has several advantages compared with other animal models, including suitability for acute and chronic brain injury studies, small body size for easy handling and testing, the need for few experimental instruments, and its relatively low cost. Although rodents have greatly contributed to our understanding of HIE over the decades, they also have several limitations, including immature brains at birth relative to humans, a rapid postnatal rate of maturation, and difficulties in the monitoring of multiple organ functions (Roohey et al., 1997). In terms of the timing of oligodendrocyte lineage (OL) progression, the predominance of pre-OLs in postnatal day 2–5 (P2–P5) pups is equivalent to that of humans at 24–32 weeks of gestation (Craig et al., 2003). At P7, the brain of the rat histologically resembles that of a human fetus at 32–34 weeks of gestation or of a preterm newborn (Vannucci and Vannucci, 2005).

Although a variety of modeling strategies have been reported in rats (Table 2), three common approaches using postnatal insults are currently used to produce rat HIE models: (1) the combination of ischemia with systemic hypoxia (Zhou et al., 2011); (2) ischemia reperfusion alone or combined with systemic hypoxia (Renolleau et al., 1998); and (3) lipopolysaccharide (LPS) administration together with the first method referred to above (Wang et al., 2010). Apart from the aforementioned postnatal insults, intrauterine hypoxia or intrauterine infection by LPS administration is also used

Table 2: An overview of rat models to study perinatal brain injury.

Insulting time	Animal models	Primary histological changes	References
SD rat, P7	UCAL + hypoxia (FiO_2 8% for 2–3.5 h)	Ischemic neuronal changes in ipsilateral cerebral cortex, corpus striatum, hippocampus and thalamus, and periventricular white matter over 90% of pups	Rice et al., 1981
SD rat, P7	BCAO + hypoxia (FiO_2 6.5% for 1 h)	Severe cortical infarcts in 97% of animals	Schwartz et al., 1992
SD rat, P10	BCAO + hypoxia (FiO_2 8% for 1–15 min)	Pyknotic cells in cortical regions, damaged pyramidal cells in the CA1 layer at 28-day recovery	Recker, 2009
Wistar rat, P2	BCAO	Rarefaction in the internal capsule in 90.9% of brains, small cerebral infarction in 9.1% of brains	Uehara, 1999
Wistar rat, P7	Permanent MCAO + transient UCAO for 1 h	Cortical infarcts in the frontoparietal cortex at 3-month recovery, robust inflammatory response	Renolleau et al., 1998; Benjelloun et al., 1999
Wistar rat, P7	Permanent MCAO + UCAO	Cortical infarcts in cortex at 3-day recovery	Wei et al., 2015
Pregnant SD rat	Hypoxia (FiO_2 10%) from G5 to G20	Focal lesions and gliosis in white matter	Baud et al., 2004
SD rat, P2	LPS (0.05 mg/kg) + UCAL + hypoxia (FiO_2 6.5% for 1.5 h)	Decreased number of oligodendrocyte progenitors and elevated microglial activation in the white matter	Wang et al., 2010
Pregnant SD rat	Maternal LPS injection (0.5 mg/kg) at G18 and G19	Increased apoptotic cells in the periventricular white matter and impaired myelination at P7	Kumral, 2007

BCAO, Bilateral carotid artery occlusion; FiO_2, fraction of inspired oxygen; G, gestational; LPS, lipopolysaccharide; MCAO, middle cerebral artery occlusion; P, postnatal; SD, Sprague-Dawley; UCAL, unilateral carotid artery ligation; UCAO, unilateral carotid artery occlusion.

to induce brain injury in fetuses (Boksa, 2010; O'Shea et al., 2013).

In 1981, Rice and co-workers developed a rat hypoxic-ischemic brain damage (HIBD) model by modifying the Levine technique in the immature animal, which then became the most widely used model in this field (Rice et al., 1981). P7 rats were subjected to unilateral common carotid artery ligation followed by systemic hypoxia with 8% oxygen delivery at 37°C for 2–3.5 h. Pups were sacrificed for examination at 50 h after the insult. Over 90% of the animals developed moderate to severe ischemic neuronal changes in the ipsilateral cerebral cortex, corpus striatum, hippocampus, and thalamus, as well as in the periventricular white matter, whereas cerebral infarcts were mainly detected along the distribution of the middle cerebral artery. This model has been utilized by a large number of investigators with minor modifications and has been proven to be of value for perinatal HIBD research (Schwartz et al., 1992; Chen et al., 2015; Cheng et al., 2015; Hattori et al., 2015). As mentioned above, the brain of the P7 rat is immature (equivalent to a human brain at 32–34 weeks of gestation). Thus, in order to better mimic the neurobehavioral characteristics and pathological changes observed in full-term human neonates with HIE, some researchers prefer to choose P10 rats. Similar to the changes observed in full-term infants, this HIE model is associated with diffuse global injury characterized by pyknotic cells in cortical regions and shrunken cells with pyknotic nuclei in the hypothalamus (Li et al., 2008; Recker et al., 2009).

Cutting off the flow of the middle cerebral artery by ligation together with unilateral middle cerebral artery occlusion induces transient focal ischemia in P7 rat pups. Brain histological examination indicates that almost all of the surviving animals present with cortical infarcts. In addition, reperfusion in these animals triggers a much more robust inflammatory response with granulocytic cell infiltration and accelerated apoptosis accompanied by enhanced caspase-3 activation within 24 h (Renolleau et al., 1998; Benjelloun et al., 1999; Manabat et al., 2003; Wei et al., 2015).

Pre-OLs, which are the dominating vulnerable cell population in the cerebral white matter, are abundant in the rat and the mouse at P2–P5. Therefore, P2–P5 pups subjected to specific insults may be more appropriate for investigating white matter injury (Craig et al., 2003). Uehara et al. (1999) reported that bilateral carotid artery occlusion alone resulted in preferential white matter injury in P5 rats, with 90.9% of the animals' brains displaying typical white matter injury. P5 Sprague-Dawley (SD) rats subjected to unilateral ligation of the carotid artery in association with 8% O_2 inspired for 2 h displayed partial formation of cystic spaces and extremely sparse myelin sheath formation in the corpus callosum at 7 days after injury (Mao et al., 2012, 2013). Prenatal hypoxia also imposed deleterious effects on the neurodevelopment of fetuses. Subjecting pregnant SD rats to long-term hypoxia (10% O_2) from gestational day 5 (G5) to G20 led to the spontaneous delivery of neonates. Focal lesions in the white matter were observed in the majority of neonatal

rats at P3, accompanied by increased lipid peroxidation and activated macrophages, indicating that gestational hypoxia may induce white matter damage in neonatal rats through an inflammatory response and oxidative stress (Baud et al., 2004).

The fact that white matter lesions are more common in offspring delivered by mothers with histological or clinical chorioamnionitis suggests that inflammatory reactions play an important role in the pathogenesis of preterm white matter lesions (Spinillo et al., 1998; Shalak et al., 2002; Herzog et al., 2015). Infectious models for white matter lesions were developed by using postnatal LPS exposure combined with subsequent hypoxia-ischemic insult in extremely immature rats. These rats exhibited more severe white matter injury compared with rats exposed to hypoxia-ischemia alone and had significantly decreased numbers of oligodendrocyte progenitors in their white matter. They also had increased microglial activation and tumor necrosis factor-α expression (Wang et al., 2010, 2016). Systemic maternal LPS administration may differ substantially from the direct administration of LPS to fetuses or neonatal rats as LPS administered systemically to the pregnant rat does not enter the fetuses and will not result in fetal endotoxemia (Goto et al., 1994). Exposure to LPS consecutively during late gestation was associated with neonatal brain injury by triggering the placental inflammatory response with excessive production of pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β, in neonatal brains with white matter lesions (Cai et al., 2000). In this model, white matter damage was detected at a variety of postnatal ages, which was indicated by microglial activation, damaged myelinization, over-activated astrocytes in the hippocampus and cortex, increased apoptotic cell death, and decreased oligodendrocyte numbers (Kumral et al., 2007; Boksa, 2010; Favrais et al., 2011; Jin et al., 2015).

Strain-specific differences in OL maturation in the three widely studied strains of rat (SD, Long-Evans, and Wistar) should be considered when modeling HIBD in rats. Compared with the other two strains, P2 Wistar rats had the highest percentage and density of pre-OLs, which are the major susceptible cell type in periventricular white matter injury. However, by P5, all three strains display similar OL maturation (Dean et al., 2011a).

Researchers also need to consider sex differences in animal models, which influence brain histology, behavioral outcomes, and response to treatment in rats (Wen et al., 2006; Smith et al., 2015). Compared with males, female neonates displayed significantly smaller infarct sizes and fewer seizures 3 days after hypoxic-ischemic insult and had less extensive brain tissue loss and behavioral deficits at the chronic stages of HIE (Smith et al., 2014; Mirza et al., 2015). Compared with females with HIBD, increased microglial activation and inflammatory response were detected in males with HIBD, indicating that hypoxia-ischemia may induce sex-specific local inflammatory reactions in the brain (Mirza et al., 2015). Sex-specific gonadal hormones may contribute to these differences. Testosterone may enhance neuronal excitotoxicity after hypoxic-ischemic insult, aggravating the deleterious consequences of early hypoxic-ischemic brain injury (Yang et al., 2002; Hill et al., 2011). The preferential activation of the caspase-dependent apoptotic pathway in females may thus afford greater protection, indicating that the activation of cell death pathways in response to hypoxia-ischemia may also be involved in the mechanisms underlying sex-related phenotypes in HIE (Hill and Fitch, 2012).

Mouse

Transgenic technology facilitates the genetic exploration of the mechanisms underlying HIE and enables the assessment of the efficacy of different experimental interventions. Transgenic technology is more mature in mice than in rats. Genetically modified mice, such as those with a knockout of the autophagy-related 7 gene (*Atg7*), C-C chemokine receptor type 2 (*Ccr2*), neuronal pentraxin 1 (*Nptx1*), or myeloid differentiation primary response gene 88 (*Myd88*), have been reported in the study of HIE (Wang et al., 2009; Pimentel-Coelho et al., 2015; Thatipamula et al., 2015; Xie et al., 2016).

The main processes used in developing murine HIBD models are similar to those used for rat models. However, because of the differences in size and physiology, several important changes have been adopted, including more elaborate surgical techniques and changes in the degree and duration of hypoxic exposure. The duration of hypoxia used to induce HIE in the mouse is remarkably shorter (35–65 min in most studies) than that used in rats (90–210 min) (Ten et al., 2003; Shen et al., 2010; Buono et al., 2015). As in rats, the susceptibility to and the severity of injury following hypoxia-ischemic insult alone or in the presence of LPS are also highly strain dependent. Rocha-Ferreira et al. (2015) found that unilateral common artery ligation combined with 30 min of hypoxia exposure induced minimal brain injury in the C57BL/6 and 129SVJ strains, whereas the brains of both CD1 and FVB mice showed more severe damage. With the exception of BALB/c mice, which only exhibited elevated numbers

of terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cells, LPS pre-sensitization together with hypoxia-ischemia resulted in substantial increases in overall brain infarction, microglial and astrocyte responses, and cell death in the C57BL/6, 129SVJ, CD1, and FVB strains.

Piglet

Compared with other animal models, the newborn piglet has several advantages as an HIE model, including similarities in brain development to that of a human fetus of 36–38 weeks of age (Dickerson and Dobbing, 1967). Newborn piglets also have similar circulation and metabolism to those of human infants. These characteristics make them valuable models for the translation of treatments for brain injury. In addition, newborn piglets are relatively inexpensive and have a large body size (1–2 kg at birth), which allows for instrumentation and magnetic resonance imaging (MRI) (Haaland et al., 1997; Cheung et al., 2011). Piglet HIE models have mainly been produced by post-hypoxia/ischemia to mimic the pathological alterations observed in human full-term infants. Three main strategies have been applied to develop HIE models in piglets (Table 3): the combination of hypoxia with ischemia induced by hypotension (Kyng et al., 2015); cardiac arrest followed by cardiopulmonary resuscitation (Guerguerian et al., 2002); and bilateral common carotid artery occlusion together with hypoxia (Broad et al., 2016).

After being intubated and ventilated, newborn piglets <24 h of age were subjected to hypoxia using a decreased fraction of inspired oxygen (FiO_2) around 4%. The FiO_2 was then adjusted to maintain a low peak amplitude electroencephalograph (EEG) of <5 μV and an upper margin of <7 μV. Mild insult induced by hypoxia for 20 min without a significant decrease in blood pressure caused no apparent damage in the cortex, cerebellum, basal ganglia, hippocampus, or thalamus. However, piglets exposed to continuous hypoxia to induce hypotension (mean blood pressure <70% of baseline) exhibited neuronal necrosis in the five regions mentioned above, most evident in the cortex, which is similar to the injury pattern observed in asphyxiated full-term human newborns (Foster et al., 2001; Kyng et al., 2015). To mimic prenatal asphyxia more closely, 3- to 7-day-old piglets were anesthetized and subjected to 30–40 min of hypoxia (10% inspired oxygen) and ventilated with room air for 5 min before 7 min of airway occlusion. Resuscitation was performed by reoxygenating, using sterna chest compressions, and administration of vasoactive agents if necessary. At 4 days after the insult, the piglets were sacrificed, and their brains were studied for pathology. Regional neuronal damage was detected preferentially in the primary sensory neocortices, the basal ganglia, and the ventral thalamus (Martin et al., 1997; Ni et al., 2011, 2012).

To determine the elements crucial in producing survivable neuropathological damage in neonatal HIE models, Björkman et al. (2006) compared constant hypoxia (fixed FiO_2 for 30–37 min) with a variable hypoxia-ischemia insult (adjusting FiO_2 according to low amplitude EEG and mean blood pressure). Although the gross neuronal injury was not significantly different between the constant and variable insult groups, the numbers of piglets with neuropathological damage were higher in the variable insult group compared with the constant insult group. The authors also observed that physiological variables, such as mean arterial blood pressure, low amplitude EEG, pH, and arterial base excess, were significantly related to neuropathological consequences.

Another piglet model of perinatal asphyxia was introduced by reversible occlusion of both common carotid arteries together with initial exposure to 12% oxygen under continuous surveillance for the detection the β-nucleotide triphosphate peak height by ^{31}P-magnetic resonance spectroscopy. The FiO_2 was adjusted to maintain a β-nucleotide triphosphate peak height of 40% above baseline for 12.5 min. Brain histological examination at

Table 3: An overview of piglet models for perinatal brain injury.

Insulting time	Animal models	Primary histological changes	References
Piglet, <24 h	Hypoxia + hypotension	Neuronal necrosis in cortex, cerebellum, basal ganglia, hippocampus, and thalamus	Foster et al., 2001
Piglet, 3–7 days	Cardiac arrest	Neural damage in primary sensory neocortices, basal ganglia, and ventral thalamus	Martin et al., 1997
Piglet, <40 h	Transient UCAO + hypoxia	Neural injury dominating in the caudate putamen and thalamus	Broad et al., 2016

UCAO, Unilateral carotid artery occlusion.

48 h after hypoxia-ischemia revealed that neural injury was particularly dominant in the caudate putamen and thalamus, with extensive vacuolation of the neuropil. In combination with magnetic resonance spectroscopy, the cerebral energy metabolism of HIE animals can be monitored in succession using noninvasive markers such as lactate/N-acetyl aspartate and lactate/total creatine ratios (Robertson et al., 2013; Alonso-Alconada et al., 2014; Broad et al., 2016).

Rabbit

Rabbits, similar to humans, undergo significant perinatal brain development, with the maturation of OLs beginning antenatally and myelin formation occurring postnatally. Rabbit fetuses subjected to hypoxia-ischemia exhibit typical white matter injury resembling human preterm encephalopathy (Buser et al., 2010). In terms of the maturation of the OL, G22 rabbits with dominant early OL progenitors are approximately equivalent to human fetuses at 22–27 weeks of gestation. The density of pre-OLs increases markedly between G24 and G25 in major forebrain white matter tracts and coincides with a significant increase in acute white matter injury after hypoxia-ischemia. This may explain the observation that there was increased acute white matter injury in rabbits subjected to hypoxia-ischemia at G25, whereas preterm rabbits at G22 displayed remarkable gray matter injury with minimal white matter injury after global hypoxia-ischemia (Yu et al., 2011). Furthermore, rabbits have flexible limbs with developed motor ability at birth, which helps in mimicking the manifestations of cerebral palsy caused by long-term HIE and in evaluating the degree of brain functional injury using behavioral testing. Rabbits are also relatively cheap and conducive to manipulation due to their mild temperament (Derrick et al., 2007).

Repetitive or sustained uterine ischemia alone or inoculated with bacteria on various embryonic days has commonly been used to produce models of preterm brain injury in rabbits (Table 4). In addition, postnatal carotid artery ligation (D'Arceuil et al., 1998) and induced cardiac arrest by asphyxia were also applied to develop brain injury in rabbits (Kohlhauer et al., 2015).

Premature rabbit models of uterine ischemia have mainly been induced by interrupting placental oxygen delivery at G21–G22 via intrauterine occlusion of the descending aorta for variable lengths of time. The incidence of stillbirths grew significantly, and the proportion of survivors with hypertonia at P1 increased to approximately 83% in animals subjected to 40 min of sustained hypoxia. Histologically, aggregation of microglia and phagocytic macrophages was observed in the corona radiata, caudate-putamen, ventral thalamic nuclei, and hippocampal formation of animals surviving for 10 days in the 40-min hypoxia group (Derrick, 2004). In contrast, repetitive hypoxia-ischemia at G29 (20 cycles of 2-min uterine ischemia followed by 1 min of reperfusion) resulted in more severe cortical damage and brain edema in rabbits, which may have been caused by increased free radical production. Yoon et al. (1997) inoculated pregnant rabbits with *Escherichia coli* at G20–G21 to generate chorioamnionitis and produced a model of white matter injury in rabbits. Twelve fetuses born to 10 pregnant rabbits in the *E. coli*-inoculated group displayed brain white matter damage, including increased karyorrhexis, rarefaction, and white matter disorganization.

Since rabbits have larger brains than other age-matched rodents, brain injury in rabbits exposed to hypoxia-ischemia is easier to monitor continuously using MRI. P9 rabbits subjected to right common carotid artery ligation followed by 10% inspired oxygen for various times displayed detectable cortical injury by MRI as early as 60 min after the insults. The deep brain injury following the hypoxic-ischemic insult presented as a decreased apparent diffusion coefficient in the basal ganglia, thalamus, and brainstem, which was predictive of cerebral palsy-specific motor deficits (D'Arceuil et al., 1998;

Table 4: An overview of rabbit models for perinatal brain injury.

Insulting time	Animal models	Primary histological changes	References
Rabbit G21–G22	SDAO for 30–40 min	Aggregation of microglia and phagocytic macrophages in basal ganglia and thalamus	Derrick, 2004
Rabbit G29	RIP (20 cycles, 2-min ischemia + 1-min reperfusion)	Severe cortical damage and brain edema	Tan, 1999
Rabbit G20–G21	Inoculated with *Escherichia coli*	Increased karyorrhexis, rarefaction, and disorganization of white matter in fetuses	Yoon et al., 1997

G, Gestational; RIP, repetitive ischemia-reperfusion; SDAO, sustained descending aorta occlusion.

Drobyshevsky et al., 2012). This indicates that MRI with different analysis methods, such as using early neuroimaging biomarkers, is capable of detecting brain injury and predicting the incidence of cerebral palsy (Drobyshevsky et al., 2007).

Sheep

Sheep, the most widely used large animals for the study of perinatal asphyxia (Table 5), have several distinctive advantages. The sheep brain is larger compared with the brains of other animal models and is thus appropriate for the installation of various equipment to monitor pathophysiological changes accompanying perinatal asphyxia. This is beneficial in reducing variation in experimental processes, thus producing reproducible HIE models. In terms of oligodendrocytes maturation, the onset of cerebral sulcation, and the development of the auditory and somatosensory cortices, the neurodevelopment of the preterm sheep fetus (0.65 gestation or 95 days) is similar to that of the 24- to 28-week human, whereas the late-gestation ovine fetus (0.9 gestation or 135 days) is comparable with the full-term human. The long gestation period for fetal sheep allows investigators to select more easily the proper times for the insult and its evaluation (Back et al., 2006). However, modeling HIE in fetal sheep is relatively expensive and requires elaborate instruments, a skilled surgical team, special intensive care units, and reliable breeders. Thus, the use of this model is confined to laboratories with advanced equipment (Back et al., 2012).

Gunn et al. (1992) reported that the occlusion of the common uterine artery of pregnant sheep at 0.9 terms for 30–60 min, alone or in combination with supplementary maternal hypoxia for 120 min, resulted in hypoxemia, hypercarbia, acidosis, and an initial hypertension in fetuses. Histological assessment at 72 h post-asphyxia revealed that around 60% of surviving fetuses showed cerebral damage, with the greatest neuronal death observed in the parasagittal cortex. In contrast, neuronal loss in the hippocampus was detected in only 30% of the survivors. The researchers also found that all surviving fetuses that had hypertension during the insult experienced neuronal loss, indicating a close association between neuronal damage and decreased blood pressure. Umbilical cord occlusion (UCO) at mid-gestation for 25 min resulted in more severe neuronal loss in subcortical neuronal structures, especially in the dorsolateral aspect of the basal ganglia, the cornu ammonis regions of the hippocampus, the striatum, and the thalamus, with only minor neuronal loss in the cortex (Keogh et al., 2012; Drury et al., 2014). There was also evidence of white matter injury in the UCO group, including axonal injury and the activation of microglia in the periventricular white matter and the internal and external capsules and increased apoptotic cell density in the subcortical and periventricular white matter, the caudate nucleus, and the hippocampus (Welin et al., 2005; Brew et al., 2016). Castillo-Melendez et al. (2013) induced prenatal asphyxia in Merino-Border Leicester sheep by UCO for a maximum of 10 min at 0.9 terms. All animals in the UCO group displayed widespread cell death in the cortical gray and white matter, significant loss of myelin in the corpus callosum and subcortical and periventricular white matter, and small bleeds within the basal ganglia. Hematoxylin and eosin staining demonstrated the presence of cystic lesions in cortical gray and white matter accompanying by inflammatory infiltration in half of the UCO brains.

To explore the neurological effects of multiple insults *in utero*, Mallard et al. (1993) developed a model

Table 5: A summary of sheep models for perinatal brain injury.

Insulting time	Animal models	Primary histological changes	References
Fetal sheep, 125 ± 4 days	CUAO + hypoxia	Neuronal death primarily in the parasagittal cortex, less in hippocampus	Gunn et al., 1992
Fetal sheep, 132 days	UCO (a maximum of 10 min)	Widespread cell death in cortical gray, and white matter	Castillo-Melendez et al., 2013
Fetal sheep, 103–104 days	UCO for 25 min	Evident white matter injury, severe neuronal loss in hippocampus and trivial neuronal loss in the cortex	Keogh et al., 2012; Drury et al., 2014
Fetal sheep, 122–130 days	Repetitive BCAO in fetuses	Marked neuron loss in striatum	Mallard et al., 1993
Fetal sheep, 95 days	Repetitive LPS injection for 5 days	Diffuse subcortical damage, periventricular leukomalacia, multifocal necrotic lesions in the periventricular white matter	Duncan et al., 2002
Fetal sheep, 80 days	LPS administration intra-amniotically for 28 days	Axonal injury, infiltration of activated microglia in the subcortical white matter, without cystic lesions	Nitsos et al., 2006

BCAO, Bilateral carotid artery occlusion; CUAO, common uterine artery occlusion; LPS, lipopolysaccharide; UCO, umbilical cord occlusion.

of frequent episodes of brief ischemia, in which near-term sheep fetuses were exposed to a single 30-min or three 10-min brief episodes of bilateral carotid cuffing, separated by 1 or 5 h. Compared with the single 30-min episode of hypoxia-ischemia, frequent insults at 1-h intervals resulted in a different distribution of brain damage with more severe neuronal injury, which was particularly severe in the striatum. The brain injury observed after three 10-min brief ischemic insults at 5 h was milder, with slight neuronal loss involving the striatum almost exclusively, suggesting that repeated brief episodes of ischemia alter the distribution of damage. In particular, striatal damage may be a feature of multiple insults.

Several models of white matter injury have also been developed by inducing systemic endotoxemia in fetuses. Ovine fetuses received three to five intravenous injections of high-dose LPS (1 μg/kg) beginning at 95 days of gestation for 5 days and were killed for brain tissue examination 10–11 days after the initial LPS injection. Cerebral white matter injury was detected in all subjects, including diffuse subcortical damage, periventricular leukomalacia, and multifocal necrotic lesions in the periventricular white matter. There was no evidence of damage to the striatum, the gray matter, the hippocampus, or the cerebellum (Duncan et al., 2002). In addition to apparent periventricular white matter damage (Dean et al., 2009), fetuses exposed to a single LPS dose at 102–103 days of gestation also showed reductions in cortical volume and a marked decrease in the density of cortical neurons (Dean et al., 2011b). Ewes receiving intra-amniotic injections of *E. coli* LPS starting at 80 days of pregnancy for 28 days developed chorioamnionitis. The brains of their fetuses exhibited infiltration of activated microglia in the subcortical white matter and axonal injury without cystic lesions, which was milder than previously reported damage in intrauterine LPS-exposed animal models (Nitsos et al., 2006).

Mallard et al. (2003) compared neuropathological outcomes in fetal sheep subjected to either systemic asphyxia or endotoxemia at 65% of gestation. Asphyxia was induced by 25 min of UCO, whereas systemic endotoxemia was produced by the intravenous injection of *E. coli* LPS (100 ng/kg). Both models presented white matter injury observed as microglial activation, impairments in astrocytes, and a loss of oligodendrocytes. Asphyxiated fetuses also displayed neuronal necrosis in subcortical regions including the striatum and the hippocampus. In contrast, LPS treatment resulted in only focal inflammatory infiltration and cystic lesions in the periventricular white matter with no neuron-specific injury.

Non-human primate

Non-human primates are usually regarded as the ideal animal model of human HIE as the placental function, fetal breathing, and somatic movements of non-human primates are the closest to those of humans. The longer survival of non-human primates also allows for the follow-up of the effects of hypoxia-ischemia on neurodevelopment for an extended period of time. However, ethical issues, advanced intensive care requirements, and high experimental costs have restricted the application of non-human primate models in the study of human diseases (Painter, 1995). Several species of non-human primates (Table 6), including baboons (Inder et al., 2004), rhesus monkeys (Adamsons et al., 1971), and *Macaca nemestrina* (Juul et al., 2007), have been used to investigate neurological disease in newborns. The differences in the physiology of closely related species should be taken into consideration when developing HIE models. In terms of neurological development markers such as somatic movements, breathing rhythms, and eye movements, a human fetus at 0.95 of full term is similar to a rhesus fetus at 0.75 of term (Painter, 1995). As determined by

Table 6: An overview of perinatal brain injury of non-primate models.

Species	Animal models	Primary histological changes	References
Macaca nemestrina, near term	UCO for 12–15 min	Neurodegeneration of cortical neurons and gliosis in the thalamus at 4 months of life	Juul et al., 2007
Rhesus monkeys, at birth	Cover the heads + UCO at delivery	Marked damage in the inferior colliculus and brainstem	Inder et al., 2004
Rhesus monkeys	Hypotension of pregnant rhesus monkeys	Widespread cortical-tissue pale or hemorrhagic necrosis and parasagittal infarction	Brann and Myers, 1975
Preterm baboons, G125 ± 2 days	Preterm baboons were delivered at 125 ± 2 days and neonatal intensive care for 2 weeks	White matter injury mainly in the parietal and occipital lobes and subarachnoid hemorrhages	Inder et al., 2005

G, Gestational; UCO, umbilical cord occlusion.

histological examination and MRI, the neurodevelopment of preterm baboons at 125, 140, and 160 days was comparable to that of human infants at 26–28 weeks, 30–32 weeks, and full-term human infants, respectively (Coalson et al., 1999; Yoder et al., 2002; Inder et al., 2004).

To generate perinatal asphyxia, the umbilical cords of near-term *Macaca nemestrina* were clamped for 12–18 min before delivery by hysterotomy. All newborns were hypotonic at birth with attenuated EEGs, and 40% of the animals had seizures within the first 30 h of life. The brain histological examination at 4 months of age revealed neurodegeneration of cortical neurons and gliosis in the thalamus (Juul et al., 2007; Beckstrom et al., 2011). Inder et al. (2005) developed a primate preterm model with remarkable predominance of cerebral white matter injury similar to that found in preterm human infants without the use of experimental interventional insults (infection, hypoxia, or ischemia). Preterm baboons were delivered at 125 ± 2 days (term = 184 days) by hysterotomy and received neonatal intensive care therapy resembling that for human preterm infants for 2 weeks. Fifty percent of the prematurely delivered baboons exhibited white matter injury, which was the most common neuropathology in this model and ranged from small patches of reactive astrocytosis to more extensive damage, which was manifested by activated microglia, small cystic lesions, and endothelial hypertrophy. Subarachnoid hemorrhages, the second most common injury, were found in 38% of the premature neonatal baboons. In addition, hemorrhages were also detected in the lateral ventricles, the germinal matrix, and the white matter.

Studies of brain injury induced by experimental asphyxia in rhesus monkeys are categorized into four different patterns in terms of associated cardiovascular and blood gas alterations (Myers, 1969, 1972; Brann and Myers, 1975; Inder et al., 2004). Total asphyxia, produced by covering the heads and clamping the umbilical cords of term rhesus monkeys at delivery, resulted in marked damage in the inferior colliculus and brainstem. In the partial ischemia model, monkey fetuses were subjected to partial asphyxia caused by hypotension in the mother. The fetal brains exhibited widespread pale or hemorrhagic necrosis in the cortex and parasagittal infarction. When respiratory gas exchange of the fetus was diminished gradually and held for long periods of time, the oxygen content of the fetus fell significantly in the absence of hypercapnia and acidemia, which resulted in selective white matter injury similar to that detected in juvenile monkeys exposed to cyanide or carbon monoxide. The final pattern of injury results from partial asphyxia in association with total asphyxia in fetal full-term monkeys. Animals subjected to severe partial asphyxia and a relatively short duration of total asphyxia experienced damage focused in the basal ganglia and the cerebral cortex, whereas those exposed to prolonged episodes of total asphyxia had more noticeable brainstem abnormalities. Overall, both the clinical and pathologic findings presented in these full-term monkeys subjected to a range of intrauterine asphyxia severity remarkably resembled those noted in perinatally damaged humans.

Conclusions

The conditions used to create experimental animal models are less variable and easy to control, which benefits the study of single factors involved in the pathogeneses of HIE. Thus, various animal models have been widely used to investigate the mechanisms and pathophysiological changes underlying HIE over the past few decades. It is crucial to choose appropriate animal models according to the research purpose. Small animal models, such as rodents, are advantageous for the exploration of the underlying mechanisms and biochemical consequences of perinatal brain injury. However, when evaluating treatment regimens preclinically, investigators favor large animals with similar cardiovascular systems to humans that also provide better access to ongoing physiological parameters during *in utero* or antenatal events. Although numerous animal models of HIE have been developed, no animal model completely mimics the alterations seen in human HIE due to the complicated changes during the perinatal period and the complexity of brain injury in humans. Brain damage is time and duration dependent in neonates because of their rapid brain development and changes in their biochemistry, physiology, and cardiovascular system (Yager and Ashwal, 2009). This makes it harder to develop ideal HIE animal models. To better understand the pathogenesis of HIE and explore effective and safe therapies for HIE, it is likely that in the future, great efforts will be put into further optimization of existing models and the development of new models. The age of the experimental animals, as well as modeling strategies and sampling times, needs special attention.

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